

Evolutionary and molecular genetics of regulatory alleles responsible for lactase persistence

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Submitted for the Doctor of Philosophy degree at University College London

July 2014

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I, Anke Liebert confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Persistence of lactase into adulthood varies in frequency worldwide and is attributable to several different single nucleotide changes in an enhancer of the *LCT* gene. One of these is at particularly high frequencies in Europeans and several others have been found elsewhere. However, information about their worldwide distribution is patchy.

2056 DNA samples from populations of Europe, Asia and the Middle East were sequenced to examine the distribution of allelic variants of the *LCT* enhancer region. It was confirmed that -13910*T is also the predominant allele around the periphery of Europe, and that this allele extends as far as the South and East of the Arabian Peninsula. Other alleles appear to have spread out of Africa or Arabia and most variation was found in the Middle East. No new common alleles were found that were likely to be causal.

In previous studies four lactase persistence associated nucleotide substitutions (-13910*T, -14010*C, -13915*G and -13907*G) have been studied functionally. In this thesis four additional enhancer alleles were examined using enhancer/promoter construct transfections and electrophoretic mobility shift assays. Three enhancer variants alter transcription factor binding *in vitro* and/or reporter-gene expression. Bioinformatic tools and specific antibodies were used to assist in identifying the transcription factors involved. The results show that different mechanisms lead to a disruption of the normal down-regulation of lactase in adult life.

Known haplotypic markers were assessed on an overlapping set of samples and a greater number of flanking markers was typed in an extended region of 1.8 Mb around *LCT*, in order to chart the evolutionary relationships and extent of historic recombination of the chromosomes carrying the derived alleles, as well as those that do not. All of the functional alleles tested have longer extended haplotypes than their ancestral counterparts, but so also does the derived variant at position -958, a haplotype marker for the B haplotype. The finding of an extended region of high linkage disequilibrium in all populations, and an extended B haplotype, is discussed in relation to the methods to study selection.

Because phenotypic studies suggest missing functional variants, the immediate promoter and a part of intron 2 of *LCT* were also selected for sequencing. No serious candidates were found in intron 2. Two alleles in the immediate promoter were studied by transfections but there was little evidence for any *in vitro* effect of these variants.

Acknowledgements

First of all I would like to thank my supervisors Professor Dallas Swallow and Professor Mark Thomas for giving me insights in their quite complementary methods of approaching the field of genetics. A special thank you goes to Professor Dallas Swallow for her continuous support and inspiring discussions as well as her patience and trust throughout this thesis.

I am very grateful to our collaborator Professor Jesper Troelsen for making my time in Denmark an enjoyable experience, for sharing his expertise in the world of proteins and introducing me to the Danish mentality. I would also like to thank Tine Jensen, Thomas Danielsen, Anders Krüger Olsen, Lotte Laustsen, Lotte Bram, Mette Juel Riisager and Sisse Marie Schmidt for their help and good company in Copenhagen and Roskilde.

Thanks to all the present and past people from the Darwin first floor office(s): Nic Montalva (for the constant coffee supply), Rosemary Ekong (for her special pieces of advice), Mirna Kovacevic, Kate Brown, Anna Rudzinski, Adrian Timpson, Heather Elding (hmm, those cakes), Bryony Jones, Ru Bains, and Chris Plaster for being around and brighten up my days in the office, especially with the sometimes mad conversations, within and outside of UCL.

Thanks to the wonderful LeCHE group for all the experiences, thoughts and adventures and the nice boat trips. A special thank you goes to Pascale Gerbault for being who she is and all the things we have shared.

A big thank you goes to Mari-Wyn Burley and Ranji Arasaretnam for their support in the lab and to Pascale Gerbault, Winston Lau and Yuval Itan for their contributions of programming work for this thesis.

Funding for this thesis was provided by the EU Marie Curie FP7, and the Annals of Human Genetics. I would also like to thank the sample donors and collectors of the G.E.E. DNA collections made available by DS and MT as well as Neil Bradman, Rosemary Ekong and Andres Ruiz-Linares.

A special thank you to my friends Kathrin Büttner, Sabrina Dettmer, Anique Lauber, Johanna Kirchhoff, Alice Albers-Westphal, Madlen Marschka, Caro and Pierre Bernoth and many more for all the good times outside of academia and helping me fill up my energy tanks when needed.

Most of all, I wish to thank my family: My parents Hannelore and Gerd, for their continuous encouragement and understanding, and my two beloved men, Maxim and Ben. Maxim for his support and patience, especially through the last months of this PhD, and both for all the nice disruptions and for making me smile almost every day.

Abbreviations/Glossary

bp	base pair
BP	before present
cal BC	years before Christ, from calibrated C ¹⁴ data
cDNA	complementary DNA
CEPH	Centre d'Etude du Polymorphisme Humain
ChIP	chromatin immunoprecipitation
CNV	copy-number variation
DNA	deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EHH	extended haplotype homozygosity
EMSA	electrophoretic mobility shift assay
GWAS	genome-wide association studies
HWE	Hardy-Weinberg equilibrium
HT	haplotype (combination of alleles at adjacent locations (loci) on a chromosome that are inherited together)
ID	identification
kb	kilobase
kDa	kilodalton
<i>LCT</i>	lactase gene
LD	linkage disequilibrium (allelic association)
LDU	linkage disequilibrium units
LeCHE	Lactase persistence in the early cultural history of Europe
LP	lactase persistence
LNP	lactase non-persistence
LPH	lactase-phlorizin hydrolase
Mb	megabase
<i>MCM6</i>	minichromosome maintenance complex component 6
mRNA	messenger RNA
mtDNA	mitochondrial DNA
OMIM	Online Mendelian Inheritance in Man
PBS	phosphate buffered saline

PHASE	software that determines the chromosomal phase (the maternal or paternal chromosome on which the alleles lie in heterozygous individuals) by Bayesian inference
PCO	principal co-ordinates
PCR	polymerase chain reaction
RFLP	restriction fragment length polymorphism
RNA	ribonucleic acid
SDS	sodium dodecyl sulfate
SNP	single nucleotide polymorphism
STR	short tandem repeat
TF	transcription factor
UniProtKB	Protein knowledgebase of the Universal protein resource (UniProt)

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1 Introduction

Human influence on the global ecosystem has increased and has become substantial, especially in recent years, even leading to suggestions of naming a new era, the 'Anthropocene'. No doubt, human culture has modified environments, but in reverse it was the ability to adapt well to environmental changes, phenotypically and culturally, that has made *Homo sapiens* successful as a species, being able to populate most parts of the world. Human genetic diversity we see today is the result of past evolutionary processes and one example of how adaptation to a new diet and a different lifestyle of former populations has shaped present genetic variation is lactase persistence.

This thesis is concerned with the evolutionary, molecular and population genetics of the variation in regulatory sequences responsible for altering the expression of human lactase, and leading to adult lactase persistence. This introduction reviews the relevant background, and includes information about past migrations and domestication processes, previous work related to lactase persistence and the lactase gene and an overview of methods for studying genetic variation and detecting selection, followed by the aims and an overview of the thesis.

1.1 Neolithisation – a revolutionary cultural change

The European and Asian continents have been inhabited by modern humans since their dispersal out of Africa from about 50,000 years ago (reviewed in Stoneking and Krause 2011).

The Neolithic era, which marks the transition from a hunter-gatherer lifestyle to farming and hence food production, represents a remarkable change in life circumstances which is well described by the term 'Agricultural revolution' coined by Childe (1936). It broadly involved the appearance of a package of innovations, also called the 'Neolithic package' within a few thousand years, which included the domestication of plants and animals, the use of polished stone tools, the manufacture of pottery and the appearance of permanent settlements. However, these innovations developed as gradual processes and at slightly different times across the world (Figure 1.1). The boundaries between Mesolithic and Neolithic lifestyles also often overlap, as there is evidence of hunting and gathering by Neolithic societies and typical Neolithic pottery that was found at Mesolithic

archaeological sites. The stepwise development of agriculture, where domesticated plants and animals like sheep, goat, pig and cattle increasingly built food resources, allowed the settlement of larger groups of people in permanent dwellings. This in turn allowed the development of new technologies and changed the social structures within societies, presumably accompanied by an alteration of mindset of its members (reviewed in Bellwood 2008).

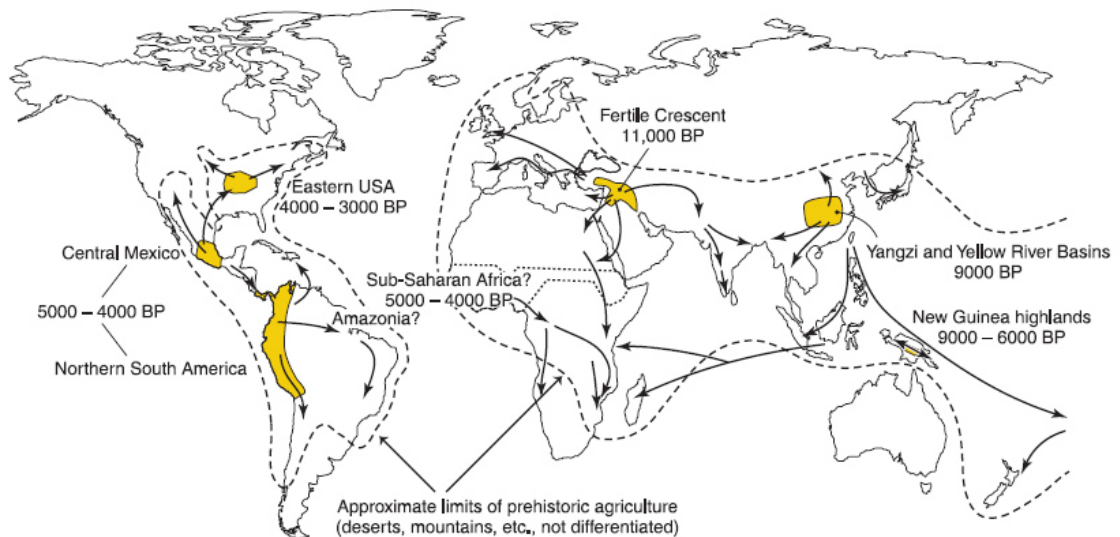


Figure 1.1: Origins and approximate expansion of agricultural systems and early farming cultural complexes from archaeological records (Diamond and Bellwood 2003).

1.1.1 The Neolithic in Europe

The process of Neolithisation relevant to Europe began around 15000-12000 years ago (Bellwood 2008) in the region of the so called 'Fertile Crescent' (Breasted 1916). This 'Neolithic core zone' extended from the Levant to Southwestern Iran via Southern Anatolia. Substantial climate changes took place at that time - the late Pleistocene to early Holocene. The temperature started to rise and the world climate became relatively stable, which would have allowed a better management of crops (reviewed in Bellwood 2008). Hunting and gathering was the only subsistence strategy across Europe before 8500 years before present (BP), until farming started to spread from the Neolithic core zone, as shown in Figure 1.2. Evidence suggests that the Neolithic culture started to spread from the Fertile Crescent to other parts of Asia and Europe and the North of Africa at approximately the same time. Two models have been suggested as to how farming could have spread into Europe. The 'cultural diffusion' or 'acculturation' model proposes that the Neolithic culture was adopted by local hunter-gatherer populations from their farming

neighbours, whereas under the ‘demic diffusion’ model the Neolithic lifestyle spread with the migration of farmers into new territories (Ammerman and Cavalli-Sforza 1984). In fact, it is likely that the Neolithisation process was more complex, with local differences (Gronenborn 2007). Palaeogenetic data support a demic diffusion model, but also showed the coexistence of hunter-gatherer and farmer populations over longer time periods (Bollongino et al. 2013; Bramanti et al. 2009; Pinhasi et al. 2012).

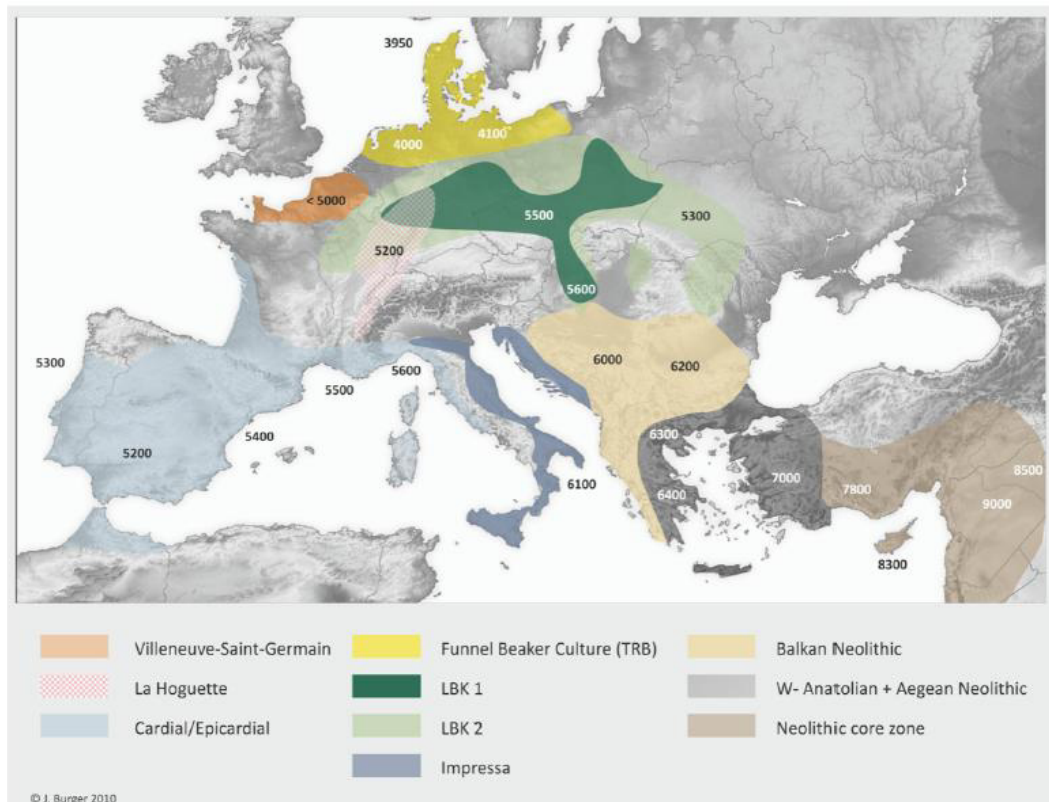


Figure 1.2: Approximate arrival dates and expansions of Neolithic cultures across Europe. Note that dates are given as years before Christ (cal BC). The picture was taken from Burger and Thomas (2011).

1.1.2 Domestication of animals

Bone assemblages from archaeological sites can reveal information about the domestication process. Besides the analyses for species affiliation, morphology, sex, age at death and cutting or cooking signs, bones can be directly radio-carbon dated and analysed for ancient DNA, which puts them in an archaeological context (Gerbault et al. 2011). Differences in morphology can be seen between the wild and domesticated forms of a species, which resulted from the break in gene flow between wild and domesticated animals and the deliberate breeding for certain traits (Vigne and Helmer 2007).

Archaeozoological evidence suggests that goat and sheep were first domesticated about 11000-10500 years ago in the Levant, followed by taurine cattle and pigs at about 10500-10000 BP. The domesticates spread rapidly to the areas of central Anatolia and Cyprus but are only found outside these areas after 8500 BP (Zeder 2008).

Archeological and archeozoological information indicated that the spread of the Neolithic culture and domesticated animals across Europe followed two routes (Figure 1.2): The first, the 'Mediterranean route' stretched along the Aegean and Adriatic Sea, to Italy, the South of France and the Iberian Peninsula. The second, the 'Danubian route' went from the Balkans via the South and West of Central Europe and further to the North. It is not clear where the routes have met but Greece, the Rhine valley and Northwest Europe are discussed as possible regions, before the Neolithic spread further to the British Isles around 6000 BP (Tresset and Vigne 2007).

The evidence for an introduction of domestic goat and sheep into Europe is relatively clear as no wild progenitors of these species have been reported. The Anatolian origin of the domesticated cattle of Central Europe is supported by ancient DNA analysis that found no evidence of mixture with the European wild cattle (Bollongino et al. 2008; Edwards et al. 2007; Troy et al. 2001). The introduced domestic pigs in contrast seem to have been substantially mixed with local wild boar as suggested by ancient DNA data (Larson et al. 2007).

1.1.3 Evidence for dairying

The management strategies of domestic herds are reflected in different patterns of slaughtering of animals (reviewed in Vigne and Helmer 2007), called 'kill off' profiles. The interpretation of these profiles can help to distinguish between culling strategies that more likely focussed on dairying or on meat production. A study on modern sheep herds in Turkey (Payne 1973) showed a massive killing of young and sub-adult males between 6-18 months, as required for meat production. Specialised dairy farming in contrast was characterised by slaughtering of very young lambs, aged below 2 months. This would of course only be true for sheep and goat since cows needed their calves to be present to stimulate milk production. Traditionally, many calves were kept until weaning in order that milk could be obtained from their mothers (reviewed in Vigne and Helmer 2007). The earliest evidence from archaeozoology for specialised breeding for milk use was found after 7500 BP in the Balkan region and 7000 BP in Central Europe. However an earlier practise of milking is very likely (Gerbault et al. 2011). Vigne and Helmer (2007) concluded from their research that milk procurement may have been at least a motivation

for the domestication of sheep, goat and cattle, which developed from stock-keeping by hunter-cultivators, to true Neolithic farmers. Because of the low milk yield at the beginning of dairying it could not be the only subsistence strategy; people must still have been hunting and gathering.

The archeometrical analysis of organic residues in pottery is another method of investigating prehistoric dairying activities. The isotopic composition of fatty acids can provide information about whether residues originated from milk or adipose fats but it cannot distinguish whether fresh or fermented milk was stored in the vessels examined (Copley et al. 2003; Dudd and Evershed 1998). With this approach, the earliest evidence for milk use was found from 8500 BP in Northwest Anatolia and Thrace (Evershed et al. 2008) and from further Neolithic sites in the Carpathian basin from around 7900-7500 BP (Craig et al. 2005), from Poland 7400-6800 BP (Salque et al. 2013), from Scotland from about 6100 BP (Copley et al. 2003) and probably Denmark around 6000 BP (Craig et al. 2011). Pot shards dated to 7200-5800 BP also revealed evidence for dairying practices in Sub Saharan Libya (Dunne et al. 2012).

However, ancient human DNA suggests that the lactase persistence was not common in early Neolithic Europe up to about 5070 BP (Burger et al. 2007; Lacan et al. 2011a; Lacan et al. 2011b; Malmstrom et al. 2010) which will be further discussed later in this thesis.

1.2 Lactase persistence (an example of gene-culture coevolution)

It has been known since Roman times (described by Hippocrates around 400 B.C.) that people show differences in their ability to digest milk. Some can handle large amounts of fresh milk without problem, others suffer from symptoms of intolerance, like abdominal pain, cramps, flatulence or diarrhoea. Much research has been done over the last 50 years, which enables us to explain these differences, although there are still unanswered questions. About a third of the world human population are capable of digesting the lactose in milk into adulthood (reviewed in Ingram et al. 2009a), a trait which is called lactase persistence (LP, OMIM #223100), as the result of a process which is seen as a remarkable example of coevolution: The interaction between genetics causing lactase persistence and the culture of milk drinking.

1.2.1 Lactase persistence status

Newborn mammals rely on milk as their first source of nutrition. Lactose is in most cases the main carbohydrate in milk, and the enzymatic activity of lactase is necessary to cleave lactose into its two absorbable component monosaccharides, glucose and galactose. Hence, lactase is present in the intestine of nearly every young eutherian mammal and its activity is high during the suckling period, except in the Pinnipedia, for example sea lions, where milk lactose is very low and no lactase is expressed (Jenness et al. 1964; Kretchmer 1971; Pilson and Kelly 1962). The enzyme is located in the brush border membrane of the small intestinal mucosa (see also 1.2.6).

After the weaning period the production of the enzyme is usually substantially reduced, to about 5-10%. This reduction is seen as the ancestral state, occurring in most humans and all other mammals tested (Ingram and Swallow 2009b; Sebastio et al. 1989). However, some of us escape this down-regulating mechanism and keep high lactase activity after childhood and throughout life, being called lactase persistent (reviewed in Ingram et al. 2009a). The mechanism for this is not fully understood yet but it was shown that lactase expression cannot be induced by prolonged intake of milk (Keusch et al. 1969).

Differences exist in the timing of the decline of human lactase. Decline can start at a young age from 1 to 5 as shown in British, Chinese and Thai children (reviewed and shown by Wang et al. 1998), whereas in others it takes up to 18-20 years to reach the lowest level of

activity as described in Finns (Sahi et al. 1983). It is not yet clear what the reason for this variation is, but it may either be due to environmental factors or other genetic influences or both.

1.2.2 The geographic distribution of the lactase persistence trait

From a European point of view, lactase non-persistence (LNP) was thought to be rare and an abnormality until the early 1960s when cases of adult hypolactasia were reported as a normal condition (Auricchio et al. 1963; Bayless and Rosensweig 1966; Dahlqvist et al. 1963). With the collection of more data on worldwide lactase persistence frequencies the picture became clearer, that lactase non-persistence is actually the more common phenotype (Flatz 1987; Swallow and Hollox 2000), in 65% of the world population (Ingram et al. 2009a).

Figure 1.3 shows the worldwide distribution of lactase persistence frequencies, which are highest in Northwest Europe, namely Denmark, Ireland and the British Isles. Lactase persistence frequency decreases as one moves towards the East and South from Northern Europe. A similar gradient has been observed in India with more people being lactase persistent in the North (Swallow 2003).

In Africa and the Middle East the picture is different, with a variable lactase persistence distribution. Some African and Arabian nomadic tribes that are milk dependent have higher lactase persistence frequencies than their neighbouring non-pastoralists (reviewed in Ingram and Swallow 2009a; Itan et al. 2010) such as the Beni Amer (pastoralists) and Donglawi (non-pastoralists) of Sudan with lactase persistence frequencies of 64% to 20% respectively (Bayoumi et al. 1982; Bayoumi et al. 1981; Holden and Mace 1997).

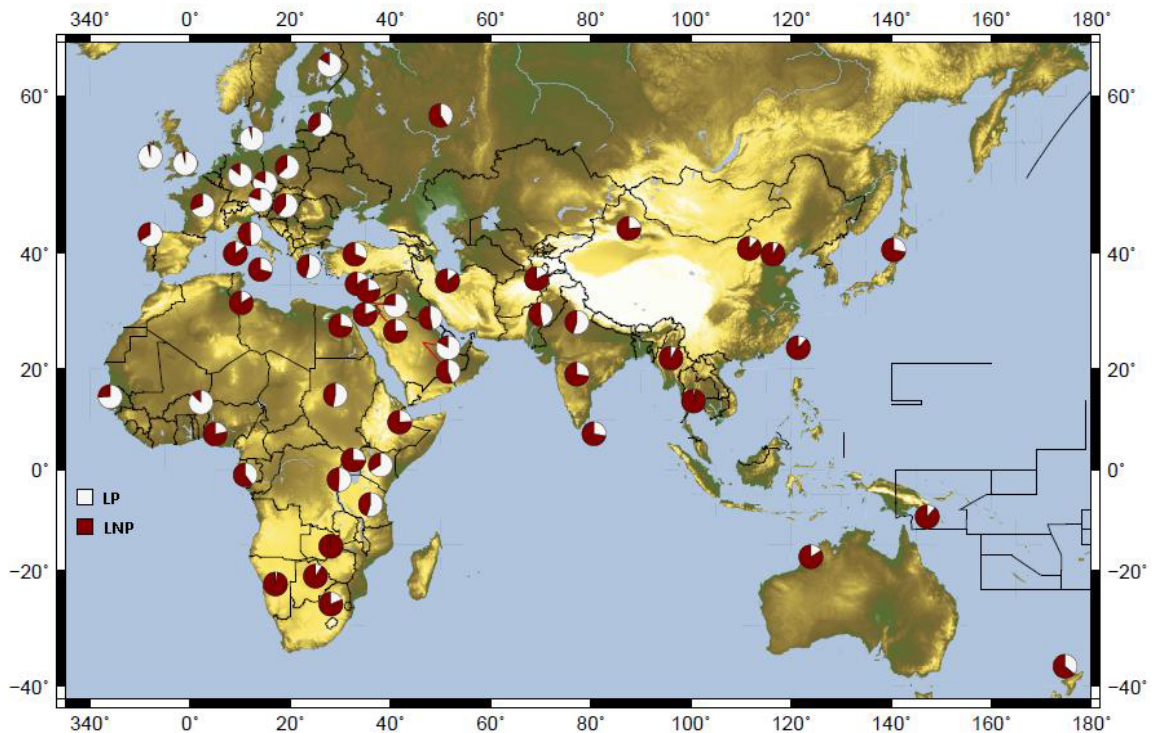


Figure 1.3: Geographic distribution of lactase persistence frequencies. White indicates the proportion of LP individuals in a population, red LNP individuals respectively. The overall frequency for each country is mostly comprised of different ethnic groups with different LP frequencies. Note that large differences in LP frequency are sometimes seen for example between Bedouin and non-Bedouin groups of Saudi Arabia and Jordan and North Indian and Indian populations. Data are taken from Itan et al. (2010).

1.2.3 Clinical definitions of lactase deficiencies

Lactase non-persistent individuals have a high probability of suffering from symptoms of lactose intolerance after consumption of a certain amount of lactose or milk, because the low level of intestinal lactase activity may not be sufficient to hydrolyse the quantity consumed. Symptoms can then occur when the undigested lactose passes into the small intestine, where it builds up an osmotic gradient leading to an inflow of water, causing diarrhoea. In the large intestine, colonic bacteria can ferment the lactose and this produces gases and fatty acids which can cause symptoms, mainly such as discomfort, flatulence and even cramps (reviewed in Hammer and Hammer 2012; Hammer et al. 1996; Hollox and Swallow 2002).

Lactose intolerance symptoms vary individually but if present they are usually recognisable within a few hours of the lactose consumption. Even quite large amounts of lactose can be in the tolerable range for some non-persistent people, as described below, while others claim to be able to take little or none. The reason for these inter-individual

differences in lactose tolerance are not completely clear but are likely be due in part to differences in gut flora composition or a different gastric emptying rate (Hertzler and Savaiano 1996; Lee and Krasinski 1998; Lomer et al. 2008; Szilagyi et al. 2004).

The consumption of fermented milk products like cheese and yoghurt can be a way of exploiting the nutrients in milk products and avoiding intolerance symptoms, as the lactose content of milk products decreases with natural fermentation (Food Standards Agency 2002; Swallow 2003). It should however be noted that some soft cheeses and commercial yoghurts contain substantial amounts of lactose. Interestingly, clinical trials have also shown that psychosomatic effects can influence the perception of symptoms (Briet et al. 1997; Peuhkuri et al. 2000).

The normal genetically determined trait of reducing the levels of lactase in adulthood (primary adult hypolactasia) is different from secondary loss of lactase due to other causes (secondary hypolactasia). Enzyme levels can be reduced when the intestinal epithelium is damaged, for example by inflammatory or autoimmune diseases like enteritis or coeliac disease. Lactase levels usually go back to normal after treatment of the primary disease and restoration of the intestinal epithelium, but this can also take longer if the patient is suffering from malnutrition, as shown for kwashiorkor ,and may in some of those cases even become permanent (reviewed in Dahlqvist and Lindquist 1971; Villako and Maaroos 1994).

Another very rare condition called 'congenital lactase deficiency' (CLD, OMIM #223000) describes the absence of lactase from birth. This autosomal recessive disorder can be fatal for a new born child if the condition is not identified straight after birth and is attributable to mutations in the coding sequence of the lactase gene (*LCT*, see also 1.2.5) (reviewed in Jarvela et al. 2009; Kuokkanen et al. 2006).

It has been suggested that lactase persistence status plays a part in a number of diseases of complex aetiology. The influence of lactase non-persistence on osteoporosis has been discussed, since an avoidance of milk products as a rich source of easy absorbable calcium, without the supplementation of calcium from other dietary sources, may contribute to a loss of bone mass and therefore an increased risk for osteoporosis (Lomer et al. 2008; Mattar et al. 2012). Several studies also have tried to relate milk consumption and lactase persistence/non-persistence to the risk of developing cancer (Larsson et al. 2006; Meloni et al. 1999; Shrier et al. 2008), metabolic syndromes (Almon et al. 2010; Corella et al. 2011; Enattah et al. 2004; Meloni et al. 2001) or gastrointestinal syndromes (Hollox and

Swallow 2002; Lomer et al. 2008; Villako and Maaroos 1994). However, a clear effect of lactase persistence/non-persistence could mostly not be confirmed and confounding effects such as mixed ancestry of the individuals tested are likely to have caused false positive associations.

1.2.4 Diagnosis of lactase persistence and non-persistence

Because lactase is restricted to the intestine, the only way of determining lactase persistence status directly is to take an intestinal biopsy to measure lactase enzyme activity. Ideally the measurement should be made in relation to another brush-border disaccharide such as sucrase or maltase, which together with histology will allow exclusion of secondary hypolactasia (reviewed in Swallow and Hollox 2000). The mRNA levels of the human proximal jejunum were shown to correlate well with the lactase persistence/non-persistence phenotype (Escher et al. 1992; Fajardo et al. 1994; Lloyd et al. 1992).

Population studies however require less invasive methods. Lactose tolerance tests are used to infer the levels of lactase indirectly as differences in lactose digestion, and volunteers can be categorised as digesters or non-digesters. Tests are carried out after an overnight fast and usually a lactose dose of 50 g is given to the volunteer. The physiological outcome is measured as difference between levels of blood glucose, urine galactose or breath hydrogen before and after the 'lactose drink' (reviewed in Ingram and Swallow 2009b). As glucose and galactose are cleavage products of lactose, an increase in blood glucose or urine galactose indicates a lactase persistent person.

All methods of testing are affected by a certain error rate but the most accurate test (Mulcare et al. 2004) is the breath hydrogen (H₂) test, which is now most commonly used for large scale tolerance testing. Hydrogen is one of various gases produced by the colonic bacteria when they ferment lactose. Partly absorbed through the intestinal mucosa, transported through the bloodstream and exhaled by the lungs it can be measured in the breath. In lactose maldigesters an increase of hydrogen in the breath is observed, provided that appropriate hydrogen producing bacteria are present in the gut.

The term 'lactose intolerant' is often used both by the public and professionals for people with lactase non-persistence. This is misleading since lactase non-persistent people do not always show symptoms in their everyday life; note that a relatively high dose of lactose (equivalent to 1 litre of milk) is used for the tests to clearly distinguish digesters from non-

digesters (reviewed in Ingram et al. 2009a; Sahi 1994), but that even in these tests breath hydrogen production does not necessarily lead to significant symptoms.

1.2.5 Evidence for a genetic cause and *cis* regulation

A genetic cause for lactase persistence was first proposed by Bayless and Rosensweig (1966) who examined differences of lactose intolerance in black and white Americans. Several family studies followed (reviewed in Swallow 1993), in particular one, using lactose tolerance tests on a cohort of large Finnish families, showed the strongest evidence for an autosomal dominant inherited trait (Sahi 1974). Later twin studies confirmed this pattern of inheritance with the lactase persistence phenotypes of monozygotic twins in complete concordance and the phenotype distribution of dizygotic twins matching Hardy Weinberg expectations (Metneki et al. 1984).

A trimodal distribution of lactase activity, as expected to be seen phenotypically by the combination of two different alleles for hypolactasia and lactase persistence, was first suggested by Rosensweig et al. (1967). By further examining the enzyme activity ratios (lactase/sucrase or lactase/maltase) of biopsy material this 'gene-dosage effect' was confirmed, with the result of one group being interpreted as homozygous persistent (highest lactase activity), one homozygous non-persistent (low lactase activity), and one heterozygous (with intermediate activity) (Flatz 1984; Ho et al. 1982). This also suggested that only one copy of the lactase gene is fully expressed in heterozygotes suggesting a *cis*-acting mechanism (Flatz 1984; Ho et al. 1982) and indeed Wang et al. (1995) were able to verify this: The allelic variants of two known exonic SNPs within the lactase gene were used to reveal the chromosomal origin of the corresponding lactase mRNA transcripts. Lactase persistent individuals who were heterozygous for the SNP marker in most cases showed a difference in the amount of transcript from each of the chromosomes, which would not exist, had the influencing factor been *trans*-acting. The conclusion from this observation of monoallelic expression was that the causal element must indeed be *cis*-acting, being located within or adjacent to the gene.

The lactase coding gene itself, *LCT* was first characterised by the group of Mantei (1988) who investigated the cDNA sequences of the gene of humans and rabbits. They deduced a 5 domain structure of the enzyme and a primary translation product of 1927 or 1926 amino acids. They found 17 coding regions (exons) and further conclusions about the structure, evolution and processing of the gene were made, as described below. Boll et al.

(1991) further examined the fine structure of the gene and compared sequences of persistent and non-persistent individuals (see below).

LCT was mapped to chromosome 2 and more specifically to the region 2q21 (Harvey et al. 1993; Kruse et al. 1988) and additional information about the sequence of *LCT* was collected within the work of the International Human Genome Sequencing Consortium (Consortium 2004) and with subsequent annotations of chromosome 2 (for example Hillier et al. 2005) which describe the gene to have a length of about 49.3 kb and being transcribed from the reverse strand of the chromosome.

1.2.6 The lactase-phlorizin hydrolase enzyme (LPH)

Lactase, also called lactase-phlorizin hydrolase, is a β -galactosidase and shows two distinct enzymatic activities working at different pH and temperature optima (Arribas et al. 2000; Naim et al. 1987; Skovbjerg et al. 1981; Wacker et al. 1992; Zecca et al. 1998). The first, lactase (EC 3.2.1.108), hydrolyses lactose and other β -galactosides such as cellobiose, -triose and -tetrose and has very low activity against cellulose. The second activity, phlorizin hydrolase (EC 3.2.1.62), cleaves the glucose from the calchone, phlorizin and other β -glycosides with large hydrophobic alkyl chains such as aryl- or alkyl- β -glycosides (reviewed in Skovbjerg et al. 1982; Troelsen 2005). Interestingly, studies revealed that lactase activity can be inhibited by phlorizin whereas phlorizin hydrolysis is unaffected by lactose presence (Leese and Semenza 1973; Skovbjerg et al. 1981). Phlorizin hydrolase is active in all vertebrates, whereas lactase seems to be specific for mammals.

The glycosyl and lactosyl ceramides of the fat globules in milk were shown to be substrates for phlorizin hydrolase and also cleaves the flavonoids-(Day et al. 2000; Nemeth et al. 2003). Dietary sources for flavonoids are mainly apples, onions, tea and red wine (Ehrenkranz et al. 2005).

In the human small intestine lactase is mainly expressed in the mid- to lower jejunum (Newcomer and McGill 1966; Skovbjerg et al. 1981). Lactase activity is generally not present in the stomach or colon, however, some mRNA was detectable in the fetal human colon and in the rat colon during the early postnatal period (Freund et al. 1990; Wang et al. 1994).

During cellular development from stem cells, epithelial cells migrate from the crypts up the villi and differentiate (as shown in Figure 1.5). In the enterocytes of the crypt/villus junction lactase mRNA expression but no protein is detected, which changes as the cells

migrate up the villus, where the protein is also present (Freund et al. 1995). It was earlier shown that lactase protein expression is highest at the middle of the villus, and decreases again towards the villus apex (Skovbjerg et al. 1981).

A number of post-transcriptional processes were shown to be necessary to form the final transmembrane protein from the LPH mRNA transcript: In humans, the mRNA of 6279 bp (Ensembl database, build 37) encoded by *LCT* is translated into a precursor protein of 1927 amino acids. This pre-pro LPH consists of a putative signal peptide of 19 amino acids, a large pro region of 849 amino acids, and an element of 1061 amino acids forming the main (extracellular) part with both active sites, a hydrophobic membrane anchor domain and a short hydrophilic cytoplasmic tail (Figure 1.4). The pre-pro LPH shows an internal 4 fold homology which is suggested to have evolved in two gene duplication events, each being homologous to a β -glycosidase unit. The first two homologous domains (I and II) are found in the pro-protein segment, the other two (domains III and IV) in the mature protein (Mantei et al. 1988).

Several post-translational modifications take place during LPH maturation (see also Figure 1.4). First, the signal peptide necessary to translocate the protein through the membrane of the endoplasmic reticulum (ER) is removed proteolytically. The resulting pro-LPH becomes N-glycosylated and forms homodimers (Grunberg and Sterchi 1995), an essential step for transport capacity and later enzyme activity (Naim and Naim 1996). The pro-region of the molecule seems to be crucial for folding and dimerisation of the protein and intracellular transport (Jacob et al. 2002; Naim et al. 1994; Panzer et al. 1998). The pro-region and domain III need to interact together for the correct folding of the 'lactase domain' IV, which in turn elevates the enzymatic activity of domain III (Behrendt et al. 2010). The pro-region is cleaved off in the Golgi apparatus after O-glycosylation has taken place (Naim and Lentze 1992; Naim et al. 1987). An extracellular cleavage of the mature transmembrane protein by luminal trypsin follows, to give rise to its final and active form (Jacob et al. 1996; Wuthrich et al. 1996).

The C-terminus of LPH is anchored to the surface of the enterocytes of the microvilli extending into the gut lumen. The two active sites are located in each of the domains of the mature 160 kDa enzyme, one for phlorizin hydrolase activity in domain III at position Glu1273 and the one for lactase activity at Glu1749 in domain IV (Arribas et al. 2000; Naim et al. 1987; Wacker et al. 1992; Zecca et al. 1998).

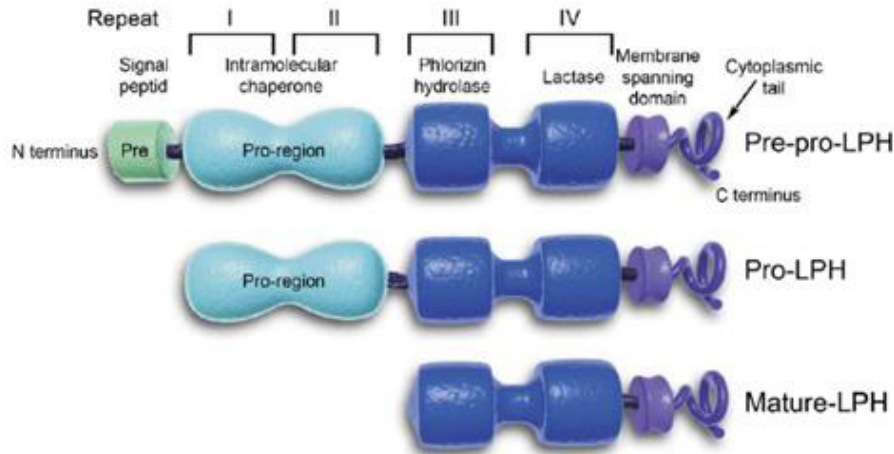


Figure 1.4: Structure of the LPH protein and its modification during the maturation process (Troelsen 2005). During post-translational processing the signal peptide and the pro-region, shown to have chaperone function, are cleaved off the 5 domain Pre-pro-LPH. The mature LPH protein consists of two domains, with phlorizin-hydrolase and lactase activities and is C-terminal anchored to the microvillus membrane.

1.2.7 Lactase expression during development - the ancestral state

Lactase down-regulation happens at a characteristic point of the mammalian development, depending on the species studied. The same seems to be true for the developmental rise of lactase activity. For example lactase mRNA expression is low in human foetuses but increases around the time of birth (Wang et al. 1998). Rodents, on the contrary, reach the maximum level of lactase activity around 3 days postnatally as their intestine is not completely matured at birth (Klein 1989).

During the post-weaning decline of lactase, a time when mammals switch their diet from milk as main food source to plants and meat, other enzymes necessary for plant digestion such as sucrase-isomaltase and maltase, are up-regulated in rodents, whereas these enzymes are already present in humans before birth (reviewed in Heitlinger et al. 1991; Troelsen 2005). This shows that although there are similarities in the developmental regulation of lactase expression in humans and other mammals, some patterns are different and the reasons for that are not fully understood.

1.2.8 Transcriptional regulation of the lactase promoter

Differences in a *cis*-acting element were suggested to be responsible for the inter-individual differences in lactase expression (as explained in section 1.2.5). Functional studies were performed to identify critical regulatory elements, first looking at the immediate promoter region of lactase. This region within the first 150 bp of the *LCT*

transcription initiation site was shown to be highly conserved between rat, mouse, pig and human, stressing its importance. Besides the TATA box, several *cis* elements in the form of binding sites for different transcription factors were identified in this region. HNF-1 α , Cdx-2 and GATA factors were shown to influence the activity of the *LCT* promoter of all these species in Caco-2 cells (reviewed in Troelsen 2005). Caco-2 is a colonic carcinoma cell line and the only human cell lines known both to spontaneously differentiate in an enterocyte like manner (Pinto et al. 1983a) and to express lactase and other small intestinal proteins as normally expressed in the small intestine when grown to confluence (Chantret et al. 1988; Hauri et al. 1985).

Although this region of the promoter seems to be important, it only directs transcription at a relatively low level in Caco-2 cells and a region further upstream was suggested to be necessary for full lactase expression (Troelsen et al. 1992). *In vivo* studies in transgenic mice showed that promoter constructs containing 1 kb of the pig or 2 kb of the rat of *LCT* promoter were sufficient for full intestinal-specific expression and the regulation of the post-weaning decline (Krasinski et al. 1997; Lee et al. 2002; Troelsen et al. 1994). The 1 kb upstream part of the promoter is similar between the mammals studied, with the exception of a disruption by two tail to tail Alu elements in humans and other primate species (Hollox et al. 1999).

1.2.9 Transcription factors involved in *LCT* promoter activity

Transcription factors shown to be involved in lactase expression are part of the network of signalling pathways and transcription factors that play an important role in the development of the intestinal epithelium, as shown in Figure 1.5. The factors Cdx-2, HNF-1 α and GATA-4 were shown to synergistically activate the lactase promoter (Mitchellmore et al. 2000; van Wering et al. 2004) as seen for sucrase-isomaltase (Boudreau et al. 2002).

A very important factor for the proliferation, differentiation and maintenance of the intestinal epithelium is Cdx-2 (Caudal-type homeobox protein 2). This transcription factor is exclusive to the postnatal intestine, mainly found in the distal small intestine and proximal colon (reviewed in Olsen et al. 2012; Silberg et al. 2000). It is involved in the transcription of many genes but the only intestinal specific factor involved in lactase expression (Troelsen 2005). As already mentioned, it binds to the immediate promoter of the human *LCT* and has been shown to up-regulate its activity when over-expressed in Caco-2 cells (Krasinski et al. 1997; Troelsen et al. 1997).

HNF-1 α and HNF-1 β (hepatocyte nuclear factor homologues 1 α and 1 β) were also shown to be important for lactase promoter activity of which HNF-1 α seems to be more activating (10 fold higher than HNF-1 β) and even more in combination with the Oct-1 transcription factor (Lewinsky et al. 2005). Both transcription factors are mainly expressed in the liver and pancreas but in other organs as well. In the gastrointestinal epithelia they are involved in the expression of several genes and essential for cell growth and differentiation (Bosse et al. 2006b; Troelsen 2005). HNF-1 α is expressed throughout the intestine (Olsen et al. 2012).

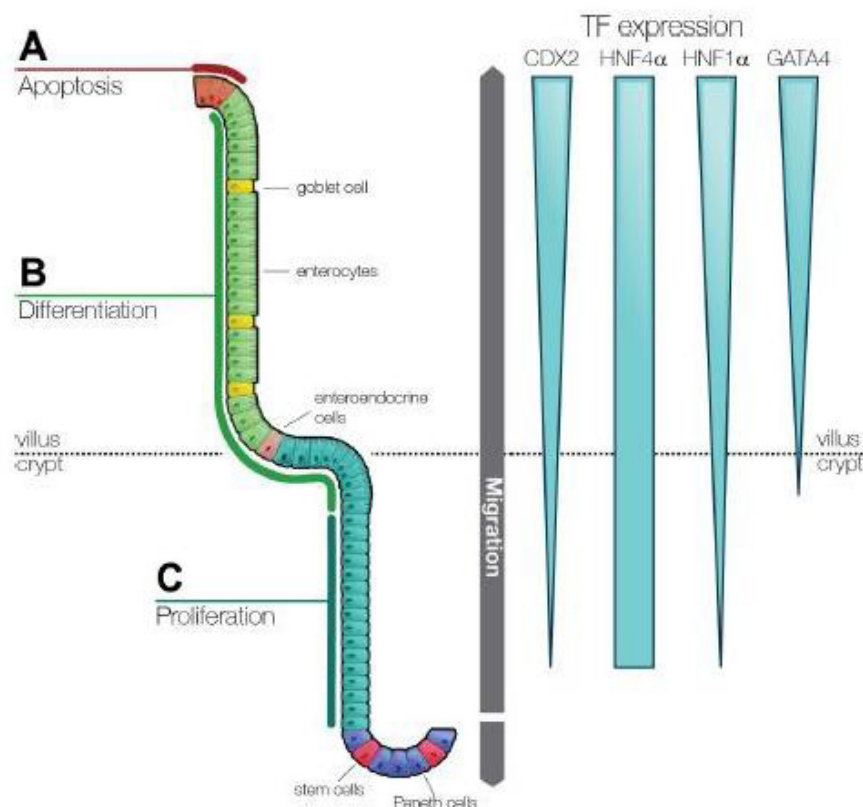


Figure 1.5: Schematic overview of the development of epithelial cells along the crypt/villus of the mammalian small intestine and important transcription factors involved (modified from Olsen et al. 2012). From stem cells, proliferating cells migrate either towards the base of the crypt or along the villus to the apoptotic zone (A). Cell proliferation (C) and differentiation (B) are mediated amongst other factors by differences in transcription factor (TF) expression: Cdx-2 and HNF-1 α are highest in the villus but already present in the crypt, GATA-4 is detectable only above the crypt and is highest at the tip of the villus. HNF-4 α expression is found equally along the crypt-villus axis.

Also GATA zinc finger family transcription factors are active in the intestine as well as in various other organs where they regulate gene expression, cell proliferation and differentiation. GATA-4, GATA-5 and GATA-6 factors have been shown to bind to the *LCT* immediate promoter (Fang et al. 2001; Fitzgerald et al. 1998) with similar affinity

(Krasinski et al. 2001). In contrast to GATA-6, highly expressed proliferating crypts of the villus, GATA-4 and -5 are mostly active in the differentiated enterocytes. Both were shown to interact with HNF-1 α to synergistically activate the *LCT* promoter but GATA-4 can also act independently (van Wering et al. 2004; van Wering et al. 2002). GATA-4 was suggested to be the main factor involved in regulating lactase activity (van Wering et al. 2002) and its influence is necessary for *LCT* expression *in vivo* (Bosse et al. 2006a). Its expression decreases from the proximal part to the distal part of the mouse small intestine, which highly but not completely correlates with the regional expression of the lactase activity which is lower in the proximal small intestine (van Wering et al. 2004).

1.2.10 Lactase persistence caused by a different enzyme?

Much research has been conducted in order to explain the full mechanism underlying the differences between the enzyme activity of lactose digesters and non-digesters. The first suggestion, that the lactase enzyme produced in adults might differ from the one in infants was not supported experimentally (Lebenthal et al. 1974; Potter et al. 1985; Skovbjerg et al. 1978). Also the structure of the precursor and mature protein was indistinguishable for both persistent and non-persistent individuals (Sterchi et al. 1990). The groups of Sterchi (1990) and Witte (1990) both found that a decreased lactase biosynthesis accounts for low intestinal enzyme levels leading to non-persistence in humans, a similar process as previously been shown for the developmental loss of lactase in rats (Jonas et al. 1985). However, it was not clear whether the variation in lactase expression was controlled by transcription or translation until finally, by comparing mRNA levels with the levels of enzyme activity, it was interpreted that it is regulated at the transcriptional stage (Escher et al. 1992; Fajardo et al. 1994; Wang et al. 1994), although this did not exclude that other mechanisms, for example alterations in mRNA stability, may influence the final enzyme level.

1.2.11 The search for lactase persistence causal variation in and around the *LCT* gene

Research focussed on regions in and around the *LCT* gene to search for causal elements responsible for lactase persistence. However, cDNA sequencing of the *LCT* coding regions (including flanking intron-exon boundaries) and the *LCT* promoter, up to 1 kb upstream of the gene, did not show 100% association of any SNPs detected with lactase persistence or non-persistence (Boll et al. 1991; Lloyd et al. 1992) nor was there any evidence for alternative splicing (Boll et al. 1991). Nevertheless several polymorphisms were found and although not completely associated they were used as genetic markers in population

studies (Boll et al. 1991; Harvey et al. 1995a; Hollox et al. 1999). 11 of these variants in linkage disequilibrium across the gene were used to define the *LCT* 'core haplotypes' spanning 60 kb. Only four haplotypes A, B, C and U were common worldwide but a greater diversity was found in African populations (Hollox et al. 2001). The A haplotype showed an association with lactase persistence and is very frequent in Northern Europeans (Harvey et al. 1998; Hollox et al. 2001). The search for a causal allele within and outside the 60 kb haplotype region continued as the A haplotype was also shown to be present in non-persistent individuals (Harvey et al. 1998; Wang et al. 1998).

Sequencing of more distant regions around the *LCT* gene identified a SNP about 14 kb (-13910 bp) upstream of the transcription initiation site of *LCT*, that was completely associated with lactase persistence in Finns (Enattah et al. 2002). It is also highly associated in other Northern Europeans, occurring on a very extended A haplotype background of about 1 Mb (Bersaglieri et al. 2004; Poulter et al. 2003). This polymorphism (-13910 C>T) is located in intron 13 of the adjacent gene *MCM6* (minichromosome maintenance complex component 6, Figure 1.6), a gene with an important role in cell cycle regulation. In contrast to *LCT*, *MCM6* revealed no inter-individual differences in expression or tissue specificity (Harvey et al. 1996). Another polymorphism, -22018 G>A, was also associated with lactase persistence in the same study of Enattah and colleagues (2002). However this -22018*A always occurred with -13910*T but not vice versa and was also found in non-persistent individuals (Enattah 2002, Poulter 2003).

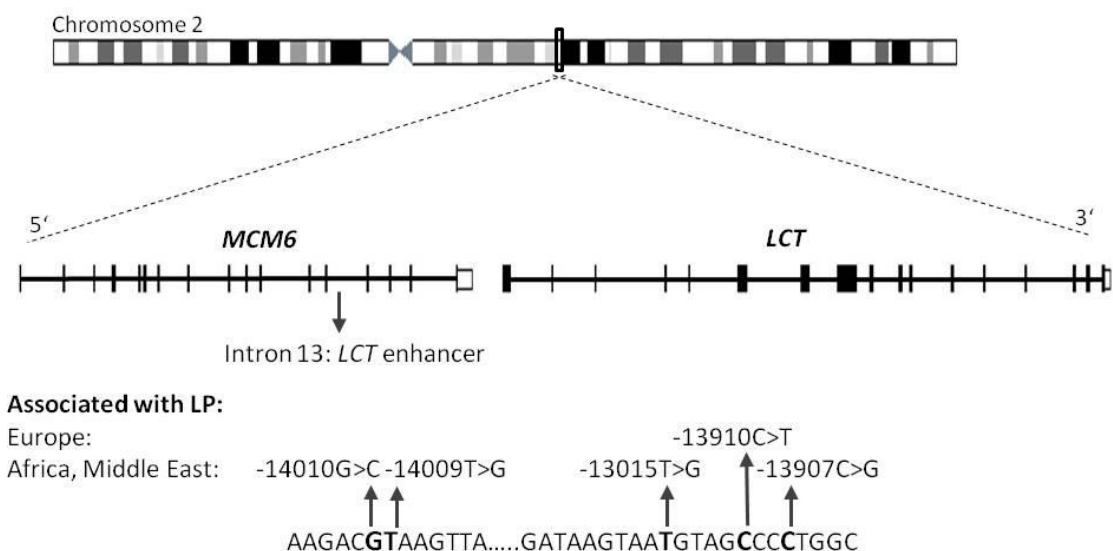


Figure 1.6: Location of the LP associated alleles in the '*LCT* enhancer' region in intron 13 of *MCM6*, upstream of *LCT*. Note that the genes are transcribed in - direction of the chromosome.

The -13910*T allele was seen as the likely cause (confirmed functionally later as described below) for lactase persistence in Europe and Asia but it did not explain the high lactase persistence frequencies of some sub-Saharan African populations or that of the Bedouins of the Arabian Peninsula where it is rare (Ingram et al. 2007; Mulcare et al. 2004). In these geographic regions other genetic variants have been detected, located very near to -13910 C>T in the same intron of *MCM6*, within a segment which has been shown to influence enhancer function (see section 1.2.12). Three of them, -13915*G, -14010*C and -13907*G, showed clear association with lactase persistence (Enattah et al. 2008; Imtiaz et al. 2007; Ingram et al. 2007; Ingram et al. 2009b; Tishkoff et al. 2007), while a further allele, -14009*G, showed suggestive evidence of association (Ingram et al. 2009b).

Other rarer alleles in this region may or may not cause lactase persistence but even if all are taken into account some lactase persistent individuals carry no enhancer allele. Itan et al. (2010) tried to address this question at a worldwide level and compared phenotypic data with predicted phenotype frequencies from all four confirmed lactase persistence associated alleles. In some parts of the world a lack of correlation between the predicted and actual lactase persistence values was apparent (Figure 1.7). This was especially the case in parts of Africa, East and South Europe and Asia where genotype data were unable to explain lactase persistence frequencies. As most of the values were interpolated and sometimes only data for -13910 C>T were taken into account, this could simply be caused by sampling problems. Nevertheless, this pattern is striking in some regions and might suggest geographic regions under the influence of a novel causal allele resulting in lactase persistence occurring at high frequency. For example in the Wolof of Senegal, where 51% lactase persistence was reported, none of the four enhancer variants had been found so far (Ingram et al. 2009b; Mulcare et al. 2004).

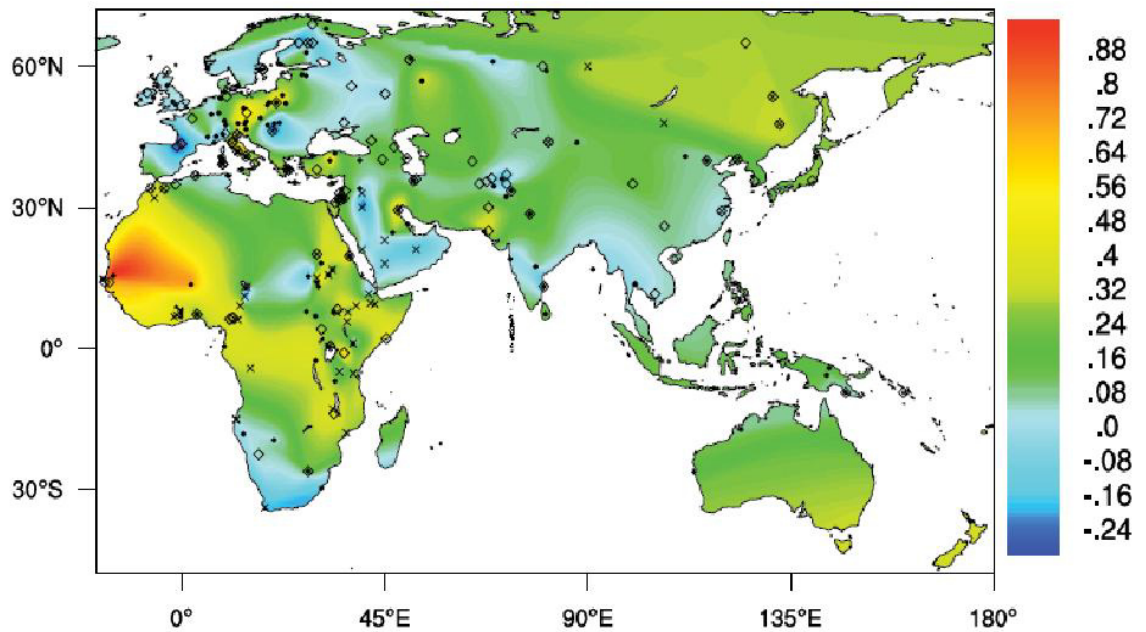


Figure 1.7: Lactase persistence genotype-phenotype correlation (taken from Itan et al. 2010). The map shows the quantitative differences between predicted LP phenotype frequencies from genotype data and observed phenotype data (both interpolated). Allele frequency data of alleles *-13910*T*, *-13915*G*, *-14010*C* and *-13907*G* were taken into account to predict the LP phenotype frequencies. For geographic regions where no phenotype or genotype data were available, values were interpolated and finally predicted LP frequency values subtracted from observed values. Colour key on the right: + values represent the genotype under-predicting phenotype at a certain location and - values over-predicting it, respectively. Data collection points: ◇-*13910*T* only, x all 4 alleles, ● LP phenotype.

1.2.12 Lactase persistence associated enhancer variants increase *LCT* expression

With the observation that *-13910*T* was completely associated with lactase persistence in a Finnish population (Enattah et al. 2002) a candidate functional allele had been found. And indeed it was subsequently shown in transfection experiments that the region around *-13910 C>T* has a transcriptional enhancer effect on the human and rat promoter of the *LCT* gene (Olds and Sibley 2003; Troelsen et al. 2003). The 450 bp human *LCT* enhancer was able to stimulate transcription about 4 to 7 fold in undifferentiated cells (2 days after transfection) and up to 50 fold in differentiated cells (9 days after transfection) compared to the promoter alone. The effect of *-13910*T* was much stronger than of *-13910*C* with a 3 to 6 times higher activity in differentiated Caco-2 cells (Troelsen et al. 2003).

One particular transcription factor, Oct-1, binds directly to the site at *-13910* but much stronger to the **T* than the **C* variant (Lewinsky et al. 2005). The function of this allele has been supported recently *in vivo* (Fang et al. 2012). The post-weaning decline of transgene

luciferase expression in mice, shown previously to be mediated by a 2 kb rat lactase promoter (Lee et al. 2002), could be prevented in mice carrying -13910*T but not in those carrying the ancestral variant -13910*C.

Subsequently other enhancer variants (section 1.2.11) have been tested for transcription factor binding: -13907*G and -13915*G showed binding to Oct-1, -13907*G with a similar affinity as the -13910*T variant (Enattah et al. 2008) but -13907*C much less (Ingram et al. 2007). Each of these variants however increases the activity of the enhancer (Olds et al. 2011; Tishkoff et al. 2007) as does -14010*C, as will be described in more detail in chapter 4.

In addition to Oct-1, further binding sites for transcription factors, namely Cdx-2, GATA-6, HNF-4 α and Fox have been identified within the 450 bp 'enhancer region' and mutation analyses revealed that all of them, except Cdx-2, are required for full enhancer activity. Further transfection experiments using co-transfection of expression plasmids for the different transcription factors themselves suggest that the interaction of Oct-1 with HNF-1 α , shown to bind to the *LCT* promoter, enables a strong enhancement of reporter gene expression and mediates the further increasing effect of the enhancer carrying -13910*T compared to the ancestral variant (Lewinsky et al. 2005).

1.2.13 The cultural practice of milking and selection hypotheses for lactase persistence

It has been observed that milk drinking behaviour is correlated with high frequency of lactase persistence and it was suggested that this is due to strong positive selection (Aoki 1986; Holden and Mace 1997; McCracken 1971b; Simoons 1970a; Simoons 1978). The selective forces that drove the increase of lactase persistence in populations with dairying practices must have acted since the beginning of animal domestication about 5000-10000 years ago.

Several theories try to explain this process. Robert McCracken using a large literature review was able to classify three groups of human cultures according to their milk product dependence (McCracken 1971a; McCracken 1971b). The first has never used dairy animals and therefore did not include lactose in their diet after weaning (e.g. Aborigines, New Guinea natives, Eskimos and the other American Indians). The second group are pastoralists who keep animals but have never used much milk or milk products as part of their lifestyle (e.g. Chinese, Thai and Philippines). Thirdly there are populations who introduced the practise of milk drinking a long time ago and therefore have a large amount

of lactose in their diet (most Europeans, many African and Middle Eastern populations and e.g. North Indians).

The close correlation of the distribution of lactase persistence with the extent the populations investigated use milk as part of their culture lead to the 'culture historical hypothesis' independently proposed by Simoons and McCracken (McCracken 1971a; Simoons 1970a). It suggests that the genetically determined trait coevolved with the practise of dairying, since with the cultural adaption to animal breeding and milk consumption the dependence of adults to milk increased. The selective pressure would act in populations consuming lactose-rich forms of milk with otherwise marginal food availability and would have favoured those who were lactase persistent, as they benefit from the nutritional value of milk, whereas fresh milk consumption may have had adverse consequences for non-persistent people. A high selective pressure of this kind would not be present in populations that process milk to sour milk and cheeses which would also allow non-persistent people to benefit from the nutrients of these products. This might explain the presence of populations with a history of dairying but low lactase persistence frequencies, as seen for example in Southern Europe (Simoons 1978).

The 'reverse cause hypothesis' is based on the same observations but describes the opposite scenario (reviewed in Aoki 2001; first mentioned in McCracken 1971a). It suggests that dairying was only adopted by populations that were already largely lactase persistent, caused by another, unrelated process. This would mean that milk drinking would not necessarily have had any selective advantage. Nei and Saitou (1986) argued that lactase persistence is so recent that selection could not have been effective enough to cause these frequencies. Drift would have been the main factor, followed by later selection (reviewed in Ingram and Swallow 2009a).

The especially high rates of the lactase persistence phenotype in Northern Europe led other authors to suggest the 'calcium assimilation hypothesis' (Flatz and Rotthauwe 1973). This focuses on milk calcium as the nutritional benefit and also the improvement of calcium absorption by lactose. Calcium is an essential bone mineral and its absorption in the gut is facilitated by vitamin D₃ (cholecalciferol). Populations in northern latitudes with lower sunlight, especially during winter, have an increased risk of developing rickets and osteomalacia due to the lack of photochemically produced cholecalciferol in their skin. In contrast to hunter-gatherers, who could balance this with a vitamin D rich diet containing marine food, early agriculturalists in Northern Europe mainly relying on cereals might

have had a problem in reaching the necessary intake of this vitamin (Cordain et al. 2012; Richards et al. 2003).

Flatz and Rotthauwe (1973) argue that drinking milk could have been an advantage for lactase persistent individuals in the Neolithic to prevent these diseases. Milk is not only high in calcium it also contains small amounts of vitamin D, and milk proteins and it has been reported that lactose additionally promote vitamin D absorption (reviewed in Ingram and Swallow 2009a).

The lack of vitamin D would surely not have been a problem for African nomads or populations from the Middle East. According to the 'arid climate hypothesis' these groups could survive better in dry regions, with limited access to water and food, using fresh milk as relatively clean and uncontaminated fluid (Cook and al-Torki 1975). For lactase non-persistent people of course this could be extremely disadvantageous if they suffer from diarrhoea and dehydration (reviewed in Gerbault et al. 2011).

Holden and Mace (1997) statistically tested the culture historical, calcium absorption and arid climate hypotheses adjusting for genetic distances between populations. They found that pastoralism alone could explain most the lactase persistence distribution, while solar radiation or aridity could not. They suggested from their maximum likelihood approach that pastoralism must have been adopted before lactase persistence became frequent. This is also supported by palaeogenetic and archaeological data as mentioned in section 1.1. Ancient DNA samples from Meso- and Neolithic periods of Europe show the absence or very low frequency of *-13910*T* implying that it was very rare or absent in early Neolithic (Burger et al. 2007; Lacan et al. 2011a; Lacan et al. 2011b; Malmstrom et al. 2010). Pottery found in Western Turkey from as early as about 8500 BP contained preserved milk residues, which would also support the idea that milk was used before lactase persistence reached high enough frequencies (Evershed et al. 2008). However, the method used cannot distinguish residues from fresh or fermented milk.

There are however some groups of people who have relatively low lactase persistence frequencies like the Dinka and Nuer from Sudan and are traditional camel or cattle herders and drink fresh milk like the Somali in Ethiopia (Ingram 2008). Many of the non-persistent individuals drink more than 500ml of milk per day. It was proposed that an adaption of the bacterial gut flora within these people may allow them to drink large quantities of milk (Ingram et al. 2009a).

The potential role of holding cows as a 'status symbol' should be taken into account too when considering the relationship between pastoralism and lactase persistence. In many hierarchical cultures keeping livestock is restricted to a 'social elite' who were the ones who had access to milk. Reproductive behaviour is also influenced by social status in such cultures (Gillis 2003; Ihara 2011; Leonardi et al. 2012; Simoons 1970a). The effect of natural selection might therefore even been greater, favouring those who were lactase persistent in higher classes who in any case have greater fecundity (Heyer et al. 2005).

An alternative selection hypothesis was proposed that is linked to the negative effect of milk consumption, considering malaria as selective force for lactase non-persistence, and suggesting that lactase persistence was the ancestral state (Anderson and Vullo 1994; Meloni et al. 1996). Anderson and colleagues argue that without milk riboflavin a mild deficiency could develop in the red blood cells and as *Plasmodium falciparum* parasites are sensitive to that it would lead to milder malaria.

On the other hand an opposing hypothesis has been proposed, that selection for lactase persistence was driven by malaria. A milk diet would be protective against malaria in causing a deficiency of p-aminobenzoic acid, a pre-form of folate, and therefore impairing the folate metabolism of *Plasmodia* (reviewed in Cordain et al. 2012). However, other studies could not find evidence for any association with, the prevalence of lactase non-persistence and malaria incidence (Auricchio 1998; Meloni et al. 1998).

As will be seen below all studies using molecular data and results of modelling imply an extremely high positive selection coefficient. However, there is still an ongoing debate about the nature of these strong selective forces, which are probably different in Northern Europe from those in other parts of the world. Undoubtedly, milk is an edible source of protein and fat (also in its fermented forms) and the practice of dairying assures a constant food supply, especially in adverse situations such as crop failure. This may be particularly important for children after weaning. A selective pressure, even if normally weak could have become much stronger under extreme circumstances like famine and drought (Gerbault et al. 2011).

1.2.14 Genetic signatures for lactase persistence under selection

In summary lactase persistence was suggested to have reached its high frequency in some populations due to positive selection acting by providing an advantage to cultures who adapted pastoralism as early as the Neolithic (Aoki 1986; Holden and Mace 1997; McCracken 1971a; Simoons 1970a; Simoons 1978).

At a molecular level, evidence for selection can be detected, for example by looking at the haplotype background of trait associated alleles (see also section 1.3.2). As already mentioned, the A haplotype is the most common in Northern Europe and shows an association with lactase persistence (Harvey et al. 1998). It was subsequently discovered that the lactase persistence causal polymorphism *-13910*T* occurs on the background of this A haplotype which extends up to 1 Mb. As this long haplotype is present at high frequency, it was thought likely to be a sign of a very recent selective process (Poulter et al. 2003).

Bersaglieri et al. (2004) further examined this signal of selection more formally, by looking at population differences of allele frequencies of 101 SNPs, spanning a region of about 3.2 Mb, and the patterns of LD as assessed by extended haplotype homozygosity. They detected a regional difference of allele frequencies, with SNPs in vicinity of *LCT* (over a 1.5 Mb spanning region) showing high inter population differences, and an unusually frequent and long haplotype of the *-13910*T* carrying chromosomes. They concluded that only strong positive selection could have led to this pattern and calculated a timeframe for it to have operated between 5000-10000 years and explained their observation with the model of allelic 'hitch-hiking' proposed by Braverman et al. (1995). Alleles surrounding the causal variant 'hitch-hike' with it to high frequency, creating a region of high LD (see also section 1.3 below). For *-13910*T* this mutation event must have been so recent that time was too short to decay the allelic associations in this region by recombination (reviewed in Sabeti et al. 2006).

Studies of microsatellite polymorphisms show similar results: a reduced diversity on the *-13910*T* carrying chromosomes (Coelho et al. 2005; Ingram et al. 2009a).

Estimations of the date when the *-13910*T* dispersion process started range from 7450-12300 years BP (Coelho et al. 2005) and 7475-10250 years BP (Mulcare 2006), both calculated by using closely linked microsatellite variation, to 2188-20650 years BP (Bersaglieri et al. 2004), estimated using the extended haplotype homozygosity (EHH) approach (see also section 1.3.2.2). The African allele *-14010*C* was reported to have occurred as an unusually extended haplotype, with the age of this variant estimated to be between 1200 and 23200 BP using EHH (Tishkoff et al. 2007). It has been observed that the variant *-13915*G* occurs mainly on a C haplotype background (Ingram et al. 2009b) and its age in a Saudi population was estimated using a coalescent approach to be about 4091 (± 2045) years (Enattah et al. 2008). No dating has been done so far for *-13907*G* but

it was shown to occur on an A haplotype background, as does *-13910*T* (Enattah et al. 2008; Ingram et al. 2009b). It is evident that lactase persistence evolved independently more than once with different causative alleles.

For alleles occurring at such high frequency in many populations, these age estimates are extremely young. With drift alone this would not have been possible, certainly not for *-13910*T* and *-14010*C*. However the combination of drift and very strong positive selection for lactase persistence could have left such a pattern and indeed the selection coefficients are estimated to be very high, between 0.8 and 19% for *-13910*T* (Bersaglieri et al. 2004; Gerbault et al. 2009; Itan et al. 2009) and between 1 and 15% for *-14010*C* (Tishkoff et al. 2007).

1.2.15 Simulation models for the spread of lactase persistence

From genetic data of modern populations alone it cannot be concluded where a variant originated or migrated from. This is especially the case because time and place a genetic variant arises does not necessarily correspond to the starting point of the selection process. To obtain further insight as to when lactase persistence first occurred and how it spread, computer simulations have been applied, since they provide a useful tool to test different scenarios. These models can take into account several factors, such as modern genetic, palaeogenetic, archaeological and geographic data to inform and to test statistical models simulating the migration.

As early as 1986, Aoki developed a model to investigate the coevolution of dairying and lactase persistence in Europe with the aim of explaining the incomplete correlation between the use of milk and the presence of lactase persistence seen today (Aoki 1986). He showed that this pattern could simply be a stochastic effect and is no evidence against the culture historical hypothesis. Although a quite simple model, for example not considering migration nor effective population sizes other than 100, he suggested that the selection coefficient must have been either constantly very high (> 5%) or the coevolution must have started much earlier than 6000 years ago.

Gerbault et al. (2009) aimed to test the calcium assimilation hypothesis and added a geographic component to their simulation models. Different selection pressures by latitude combined with two different diffusion models (Ammerman and Cavalli-Sforza 1984) were tested. The demic diffusion model assumes the spread of Neolithic over Europe and gene flow between the migrating farmers and hunter-gatherer populations. In

the cultural diffusion model, only the Neolithic cultural practice would have been exchanged between hunter-gatherers and their farming neighbours, without genetic admixture between them. Gerbault and colleagues (2009) simulated the selection process for a lactase persistence associated allele (present at 1% in LBK cultures) from the beginning of Neolithic (10000 years ago) and compared that with lactase persistence frequencies from European and Middle Eastern populations today. The results obtained would fit with the demic diffusion model and the calcium absorption hypothesis (Gerbault et al. 2009). They concluded that a positive selective process would indeed have been necessary to explain the high frequency of lactase persistence in Northern Europe. In Southern Europe on the other hand the effect of genetic drift alone could explain the lactase persistence frequencies observed today.

With a more complex model, Itan et al. (2009) simulated the spread of the lactase persistence *-13910*T* allele across Europe and the Middle East over the last 9000 years, allowing for gene flow between non-dairying and dairying farmers and hunter-gatherers. They concluded from their best fitting simulations that the *-13910*T* allele first underwent selection among dairying farmers between 6256-8683 years ago. It was also concluded that the coevolution process started between the central Balkans and Central Europe (Figure 1.8). Interestingly the combination of time and region would agree with the occurrence of Linearbandkeramik culture in Central Europe. In contrast to Gerbault et al. (2009) they suggest that a selective pressure due to latitude would not have been necessary to explain the high lactase persistence frequencies in Northern Europe, although they did not explicitly test for this.

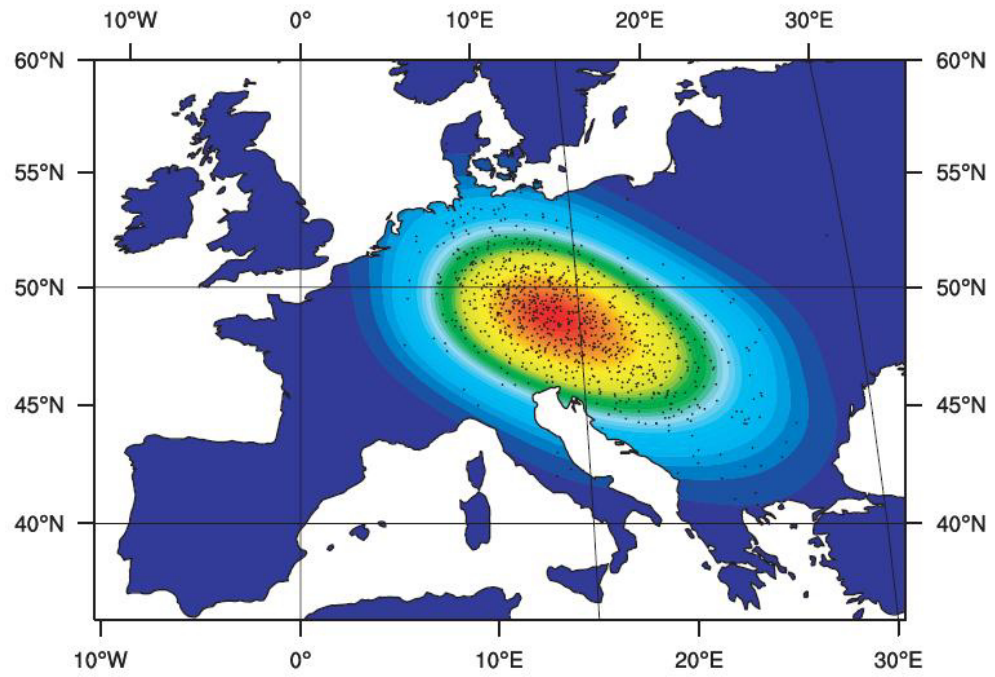


Figure 1.8: Simulated region of the origin for LP-dairying co-evolution. Best fit simulations (dots) are of highest density in the red coloured area between the Central Balkans and Central Europe (Itan et al. 2009).

1.3 Population genetics and methods to detect selection

1.3.1 Aspects of the population genetics theory

Population genetics is a research field that studies evolutionary processes leading to genetic diversity, by assessing inter and intra population differences of variation in allele and haplotype frequencies of populations. Population genetic models help to disentangle how different factors have acted over time and space to result in currently observed diversity patterns. Such factors are for example mutation, recombination, genetic drift, population subdivision, gene flow and selection. These issues are described in Jobling (2013) and presented in summary in the following sections.

New alleles are created by mutation and lead stepwise over time to other allelic forms (haplotypes). Mutation rates differ along the genome and depend on the type of mutation. Microsatellites for example have a higher mutation rate than SNPs. By knowing the mutation rate of a particular genomic region, the allele frequency can be calculated over generations with different models of DNA sequence evolution (see Jobling et al. 2013, chapter 5). Of course, only the heritable nucleotide changes in germ-cells are of evolutionary interest for these models.

In addition to mutation, meiotic recombination can generate variation and reduce the frequency of a haplotype. This process can be studied by looking at non-random correlations of alleles in populations, known as linkage disequilibrium (LD) using the D statistic. A new arising mutation is linked to all other alleles on that chromosome, being in complete LD with them (D_{\max}) and forming one haplotype. Over time LD starts to decay as recombination breaks up the haplotype and creates new allelic combinations. One useful measure of LD is D' which is D/D_{\max} . Closely linked loci on the same chromosome are less prone to recombination and selection can be detected by looking at their diversity (see below).

Whether an allele gets fixed or is lost within a population is mainly due to genetic drift. This source of variation is due to the stochastic process of sampling from one generation to the next. The extent of genetic drift relates to the size of the sampled population as described in the Wright-Fisher model (Wright 1931), if the population stays at a constant size, mates randomly and generations don't overlap. Of course, this is not a realistic model for human populations as generations do overlap, populations seldom stay at a constant in size and in large populations mating is not random. To take these influences into account and be able compare the degree of genetic drift between populations, the concept of

effective population size was developed. It specifies the size of a randomly mating population with the same degree of genetic drift as the actual population.

In most cases, new alleles will disappear from a population over time due to drift, when no selection or mutation is acting. The fixation of a neutral allele ~~only~~ depends on the population size. Considering the same mutation rate, an allele becomes fixed earlier in smaller than in larger populations and selection can accelerate this process. This means that even under neutral conditions past reductions in population size, as seen with population bottlenecks and founder effects, have a large influence on present day genetic variation.

As mentioned above, human mating may not be random because of mate choice not being independent from genetic relatedness, for example due to assortative mating (for example preference of phenotypic similar mates) or disassortative mating. Mating is also not random when a population is structured, which means it consists of partially isolated and smaller subpopulations. If isolated, members of each of these subpopulations are closer related than to individuals of all other subpopulations, due to partial genetic differentiation. If individuals migrate between these subpopulations they cause gene flow, which consequently reduces population differentiation. Population structure can be measured by the F_{ST} statistics, which shows the genetic distance between subpopulations (see also section 2.3.7).

The genetic variation of individuals defines their 'fitness', the ability to survive and reproduce in certain environments. Natural selection leads to variable reproduction of individuals and resulting in different genotypes in successive generations. Selection can operate on phenotypes at any stage of development: Survival to reproductive age (viability and mortality), success in mate attraction (sexual selection), the ability to fertilize (fertility and gamete selection), and to produce viable offspring (fecundity). These factors determine the relative fitness of an individual genotype/phenotype compared with others competing for the same resources, which can be measured as the selection coefficient (the proportion of fertile progeny relative to those without the genotype, or relative probability that a genotype will reproduce-i.e. positive selection w of $0.1 = 1 - 0.9$, where progeny of 100% (1) for carriers and 90% (0.9) for non-carriers).

Selection can act in different ways. Mutations that increase the fitness of the carrier undergo positive selection and may rise in frequency in a population, whereas mutations that reduce fitness are subject to negative or purifying selection and are possibly

eliminated from the population. However, it is important to take the combination of mutations into account when considering their effect on the fitness of a genotype. Two alleles on a diploid locus for example can have a different impact on individual fitness. A novel allele may increase the fitness of a heterozygote compared to that of both homozygotes and possibly decrease their fitness, which is called heterozygote advantage. This results in balancing selection, which favours more than one allele, increasing diversity but preventing certain alleles from fixation. Another process creating balancing selection is frequency-dependent selection, where the frequency of a genotype is crucial for its fitness and leads to higher fitness at lower frequencies compared to other genotypes.

1.3.2 Statistical tests to detect selection

Of special interest for this thesis are methods that detect natural selection. Most of the statistical methods that aim to detect selection work in considering significant deviations of the observed genetic diversity to that expected under neutrality. The neutral theory of evolution, developed by Kimura (Kimura 1968; Kimura 1983), states that most variation within and between species is neutral, which means it does not affect the phenotype or fitness of individuals and is therefore not under selective pressure. Neutral mutations are fixed in a population randomly by genetic drift and most of them stay at low frequencies or are lost from a population.

A positive selective sweep reduces the variation within a population but does not lead to decreased variation between species. Divergence data build the background to identify positive selection between species and diversity data within species. Negative selection instead tends to reduce variability within and between species (Nielsen 2005). Population genetics distinguishes between hard selective sweeps, soft selective sweeps and polygenic adaptation (Jobling et al. 2013). A recent hard selective sweep (or classical selective sweep) leads to an increase in frequency of the haplotype carrying the advantageous mutation as shown in Figure 1.9. The following paragraphs review the methods that aim to detect this type of positive selection, based on their different approaches and Figure 1.9 summarises commonly used methods. Positive selection can also act over a longer time span and favour more than one variant (soft selective sweep) that may even lay within different genes (polygenic adaptation). These forms of selection are harder to detect.

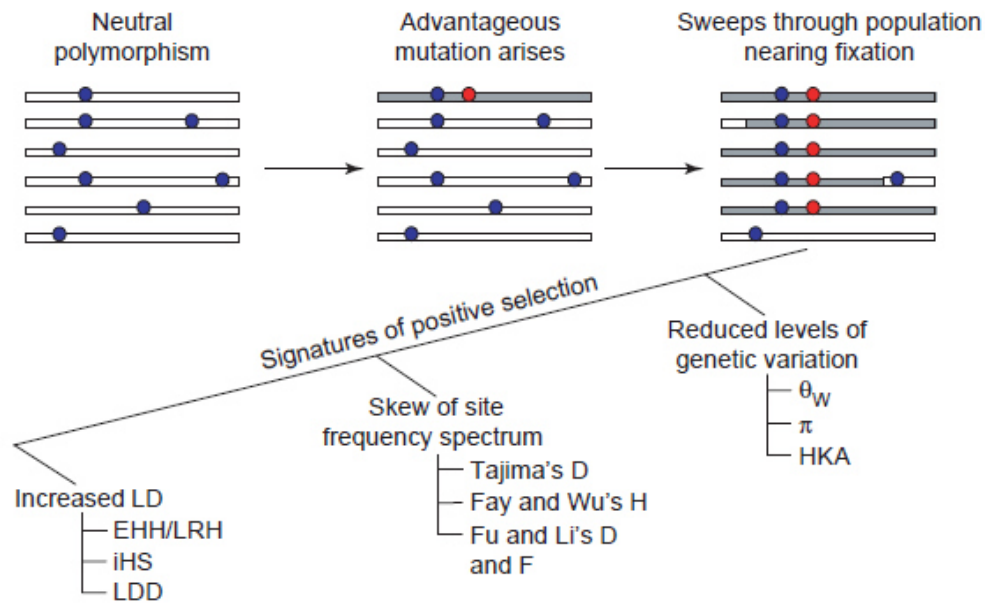


Figure 1.9: How to detect signatures of positive selection. The upper part of the figure shows how the pattern of neutral polymorphisms on different haplotypes changes when an advantageous allele (red) arises and increases in frequency and thereby drags along linked neutral polymorphisms. This process reduces variation levels surrounding the selected region, causes a skew in allele frequency distribution and increases linkage disequilibrium levels. Statistical methods commonly used to tests for these signatures are shown below, of which some are further described below. The figure was taken from Biswas and Akey (2006).

1.3.2.1 Proportion of function altering mutations

Variation in gene coding regions can be of two types: synonymous, which usually don't alter amino acids and therefore protein structure or their function and non-synonymous, which can have serious effects depending on which part of a protein they occur. By comparing the ratio of non-synonymous (d_N) to synonymous (d_S) mutations, selection can be detected. Under neutrality, a neutral non-synonymous change would increase in frequency in a population at the same rate as a synonymous change and d_N/d_S will equal one. A deleterious non-synonymous mutation will most probably be subject to purifying selection and the d_N/d_S ratio will be less than one whereas a positive selected non-synonymous change increases its frequency and d_N/d_S will be greater than one. The latter effect is expected to be stronger for species divergence data than within a species and can be tested using the McDonald-Kreitman test (McDonald and Kreitman 1991), which compares the d_N/d_S ratio at a specific locus within and between species. This test is useful in identifying selected function altering mutations in gene coding regions and it is robust against demographic factors (Nielsen 2005). However, it ignores selection of variation in gene regulatory elements in non-coding parts of the genome.

The application of a modified version of the Hudson-Kreitman-Aguade (HKA) test (Hudson et al. 1987), which compares the variation rate within and between species at multiple loci, and can be applied to non-coding sequences.

1.3.2.2 Haplotype based methods

A recent and strong positive sweep (hard sweep) can be detected when an allele under selection has increased in frequency and created a region of LD with neighbouring SNPs, which reduces genetic diversity of the neighbouring regions. Two test methods have been developed to detect this skewed allele frequency spectrums around a frequent mutation, the extended haplotype homozygosity test (EHH) (Sabeti et al. 2002) and the integrated haplotype score test (iHS) (Voight et al. 2006). EHH detects a combination of alleles that are in LD over a long region (core haplotype) and that are at high frequencies (i.e. common homozygosity). The method statistically evaluates the expansion of LD of this core haplotype. The extension of the core haplotype is compared to all other haplotypes of that region (relative EHH, rEHH) in comparing their EHH decay. The rEHH statistics also takes local recombination rates into account. A recently positive selected haplotype should show high EHH values at high frequencies, whereas a haplotype under less selective pressure or just reaching high frequencies due to genetic drift would have been more prone to recombination and mutation as this process takes longer, and would therefore show lower EHH values.

1.3.2.3 Allele frequency spectrum

Tests considering the site frequency spectrum or allele frequency spectrum take the allele frequencies at segregating sites into account. As described above, a positively selected allele tends to increase in frequency and causes a reduced frequency spectrum of other variants around it. This causes a skew in allele frequencies towards more alleles at low frequencies (Nielsen 2005). To detect this, Tajima's D (Tajima 1989) for example tests the excess of rare alleles compared to neutrality or the HKS test (Wright and Charlesworth 2004) the reduction in polymorphism levels. A further test takes the proportion of the variants at high frequencies into account that are as a result of this 'hitchhiking' process associated with each other. Fay and Wu's H (Fay and Wu 2000) test the excess of derived alleles at high frequencies.

1.3.2.4 Population differentiation

Population differentiation tests work under the assumption that selection operates differently in different populations, especially under different environmental conditions.

They can detect selection that occurred after subdivision of a population. Differences in allele frequencies might therefore be specific for a population and can be indicated by an increased level of genetic differentiation between populations, as measured for example by the F_{ST} statistic (Wright 1950), further described in chapter 2. However, inter-population differences can be highly influenced by demographic factors, such as population bottlenecks that reduce genetic diversity and F_{ST} tests should ideally be combined with other tests to get better evidence for selection.

1.3.3 Detection of different patterns of selection of *LCT*

Over the last years the availability of genome wide data and new or modified statistical approaches to scan for genomic signatures of adaptations in these datasets have identified a range of candidate genes or gene regions under positive selection. As described in above these methods mainly investigate variation patterns like allele frequency spectrums at certain regions or the reduction of haplotype diversity across populations. The *LCT* region mostly appears in these studies as strongly positive selected in European datasets (with chromosomes carrying -13910*T) as shown by long-range haplotype tests , haplotype diversity or population differentiation (Akey et al. 2002; Bersaglieri et al. 2004; Hofer et al. 2012; Sabeti et al. 2002; Sabeti et al. 2006; Voight et al. 2006). Extended haplotype homozygosity in combination with a high allele frequency was also reported for -14010*C in Kenyans, which supports the notion that a strong selective pressure has also acted on this variant (Tishkoff et al. 2007).

Recently, evidence for another type of selection acting on the *LCT* enhancer was detected in an Ethiopian dataset. Our group assessed the signatures of a soft selective sweep (Jones et al. 2013) which allows the accumulation of many rare advantageous alleles, causing the same phenotype (as described by Hermisson and Pennings 2005; Pennings and Hermisson 2006a; Pennings and Hermisson 2006b). This was inspired by the findings that several lactase persistence associated enhancer alleles were present in the Jaali and Somali groups previously investigated and that enhancer sequences of persistent people of these groups accumulated more derived alleles than those of non-persistent individuals (Ingram et al. 2009b). In taking neighbouring genetic regions of the *LCT* enhancer into account to control for demographic and mutational effects a comparison of haplotype and nucleotide diversity between lactose digesters and non-digesters revealed a clear pattern of reduction in variability only at the enhancer in the non-digesters (Jones et al. 2013).

1.3.4 Methods to study *LCT* regulation

The function of a gene is to a great extent defined by the protein it codes for. However, the regulation of gene expression ‘fine-tune’ the spatial and temporal expression of genes and can have a great impact on the phenotype in for example development processes of an organism and adaptation to environments. Inter-individual and inter-population differences in gene expression have been noticed (reviewed in Jones and Swallow 2011).

Factors that influence gene expression can be environmental, epigenetic and genetic, with elements of the latter acting *cis* and *trans* of the gene. Enhancers, silencers, promoters and insulators are such *cis* elements, which can have influence on transcription factor binding (*trans* elements) and mutations in these regions can have substantial effects on the expression of a gene. As mentioned earlier, it was shown using mRNA activities assigned to certain chromosomes that the transcription of *LCT* is regulated by a mechanism *cis*-acting to the gene (section 1.2.5) and a regulatory network of transcription factors is involved for the different lactase expression between lactase persistent and non-persistent individuals (see also section 1.2.9, 1.2.12).

Evidence for differences in transcription factor binding came from the analysis of DNA-protein interactions between proteins of the nuclear extract of the lactase expressing matured Caco-2 cells and radioactively labelled DNA oligonucleotides with sequence parts of the promoter or enhancer of *LCT* containing different mutations. The broader gene regions that are involved in the differential lactase expression were defined in transfection experiments in Caco-2 cells with plasmids containing different parts of upstream genetic regions of *LCT* (see also section 1.2.9 and 1.2.12). Details of the procedures are described in sections 2.2.6 and 2.2.7 of chapter 2. However further experimental procedures might identify other regions or elements that are involved in lactase expression, some of them are discussed in chapter 7.

1.4 Aims and overview of the thesis

The general aim of this thesis is to examine the genetic variation in and around the *LCT* gene in human populations with emphasis on Europe and the Middle East, to shed more light on the evolution of the lactase persistence. My studies also focus on how specific genetic variants can influence lactase expression.

The following specific aims were addressed in each chapter:

- To investigate the variation of *LCT* enhancer alleles in Eurasia and the Middle East (chapter 3).
- To study putative functional *LCT* enhancer alleles *in vitro* (chapter 4).
- To examine patterns of linkage disequilibrium and obtain insight into the demographic history and origin of these alleles from their haplotypic background (chapter 5).
- To explore new genetic regions for possible missing alleles for lactase persistence (chapter 6).

2 Material and Methods

2.1 DNA samples and population histories

All DNA samples analysed within this project were part of University College London, G.E.E. DNA collections, mainly of the former TCGA (The Centre for Genetic Anthropology) and of Dallas Swallow's group. The Spanish and some of the Catalan DNA samples were kindly provided by the laboratory of Professor Andres Ruiz-Linares.

Samples were collected anonymously with informed consent of the donors (or their legal guardian when under 18 years old for a collaborative project with the City of London school). The collections are covered by UCL/H ethics approvals (ULCH 99/0196, 01/0236 and UCL 2670/001) and consents were also obtained from the relevant authorities in the countries outside the UK.

The samples were obtained from unrelated individuals, being unrelated at the parental and at least grandpaternal level. Information about self declared ethnic background/cultural identity (also for parents and grandparents) and sampling locations were available in most cases. Individuals were grouped accordingly into population groups for analysis. Individual samples were classified to be part of a certain ethnicity group if their self declared cultural identity and those of both parents belong to the same group, otherwise as mixed within that country or other for that geographic region. Samples from individuals of very mixed ancestry were excluded from the study. Language classification of populations were done using the ethnologue online resource (<http://www.ethnologue.com/>) (Lewis et al. 2013).

2.1.1 Samples from Europe and Asia used for the geographic survey of chapter 3

A summary of the population samples used for chapter 3 of this thesis are shown in Table 2.1. Some of the populations, with a history particularly interesting for this thesis, are further described.

2.1.1.1 Han-Chinese

Han-Chinese do not have a long history of milk drinking; this only very recently became fashionable. According to Murdock (1967) Chinese rely on animal husbandry for up to 6-15% of their diet, but usually do not milk their animals. China was one of the centres of

domestication around 8000 years ago but the domesticated animals were non-milkable species like pigs and dogs (Cavalli-Sforza et al. 1994). Except for minority ethnic groups and regional influences of pastoralists groups from neighbouring countries, there were only two periods when the use of milk and milk products was introduced on a broader scale. Between 250 and 1000 AD Northern China was influenced by cattle-breeding societies and also 1280-1368 under Mongolian leadership (Simoons 1970b) during the Yuan Dynasty. The DNA samples were collected from Han-Chinese individuals living in Singapore. Chinese with its several forms belongs to the Sino-Tibetan languages, Mandarin is the official national language of China.

2.1.1.2 Mongols (Mongols and Khalka)

The Mongolian samples were collected in different parts of Mongolia. Dairy products are and have been of great importance to Mongols, especially kumiss, a fermented milk product.

Most of them belonged to different ethnic groups, who speak a language of the Altaic language family. Only the samples from the Khalka were enough to build an 'own population group' for analysis. The Khalka live to 76 - 85% from animal husbandry. They are pastoralists who hold cattle, water buffalo and yaks (Murdock 1967).

2.1.1.3 Tyroleans

The three Tyrolean groups sampled have a different historic background. Samples are from Bozen, Gadertal and Vinschgau and individuals speak different languages or dialects with Italian, Ladin (a mixture of German and Italian) and German respectively. The Italian speakers only recently immigrated from the South of Italy (around 1920) from different places of the country. Ladins are suggested to be the closest related to native Tyroleans and 'they acquired the typical Romance Rhaetian language during the Roman invasion of the Alps' (from the 1st century BC to the 3rd century AD). The German speaking groups from South Tyrol are believed to have immigrated around the 5th century from the North (Valentina Coia, personal communication).

2.1.1.4 Sami

The Sami are traditional hunter-gatherers living in the far North of Scandinavia and Russia where they have been indigenous for about 2000 years (Cavalli-Sforza et al. 1994). The Sami subsisted mainly from hunting and fishing until they became reindeer herders in the recent centuries and many of them live in semi-settlements most of the year (Leonard and

Crawford 2002). Samples tested for this thesis originate in Sweden. Sami is part of the Uralic language family.

2.1.1.5 Yakuts

The Yakuts, or Sakha as they describe themselves, live in the northeastern part of the Russian Federation in the Sakha Republic (or Yakutia). They are thought to originate from the Lake Baikal region and were forced to move north by the Buryat-Mongols during the 13th and 14th centuries.

Yakuts were traditional nomads and Northern and Southern Yakuts are culturally quite distinct (Wixman 1984). Northern Yakuts mainly rely on fishing, hunting and reindeer breeding whereas Southern Yakuts are cattle and horse breeders. Reindeer and mare's milk are an important part of the Yakutian diet with koumiss, their traditional drink of fermented mare's milk (Zeder 2006).

The Yakut DNA samples were divided in two sub-groups of differing origin. Samples collected in the Abyysky and Allaikhovsky Districts were grouped together as Northern Yakuts; samples from the Nyurbinsky, Ust-Aldansky and Vilyuysky Districts and from Yakutsk (phenotyped samples) were grouped as Yakuts (South). Yakut is a northern Turkic language branch of the Altaic language family.

Table 2.1. Population samples used in chapter 3, their location and language family (according to: <http://www.ethnologue.com/>).

Region	Country	Population	Language family
Northwest/ Central Europe	Germany	Germans	Indo-European
		Sorbs	Indo-European
	Ireland	Irish	Indo-European
	Netherlands	Frisians	Indo-European
	Norway	Norwegians	Indo-European
	Slovakia	Roma	Indo-European
	Sweden	Sami	Uralic
		Swedes	Indo-European
	UK	Ashkenazi-Jews	Afro-Asiatic
		English	Indo-European
South Europe	Greece	Greeks	Indo-European
	Italy	Tyroleans Bozen	Indo-European
		Tyroleans Gadertal	Indo-European
		Tyroleans Vinschgau	Indo-European
	Portugal	Portuguese	Indo-European
	Spain	Catalans	Indo-European
		Spanish	Indo-European
East/ Southeast Europe	Belarus	Belarusians	Indo-European
	Macedonia	Macedonians	Indo-European
	Romania	Romanians	Indo-European
	Russia	Erzya	Uralic
		Moksha	Uralic
		Russians Perm	Indo-European
	Ukraine	Ukrainians	Indo-European
Middle East	Cyprus	Greek Cypriots	Indo-European
	Iran	Iranians	Indo-European
	Kuwait	Kuwaiti	Afro-Asiatic
	Syria	Syrians	Afro-Asiatic
	Turkey	Anatolian-Turks	Altaic
	Yemen	Yemeni Hadramaut	Afro-Asiatic
		Yemeni Sena	Afro-Asiatic
West Asia	Armenia	Armenians	Indo-European
	Azerbaijan	Azeri	Altaic
	Georgia	Georgians	Kartvelian
Central/ South Asia	Afghanistan	Pashtuns/Afghans	Indo-European
		Tadjiks	Indo-European
		Uzbeks	Indo-European
	India	North Indians	Indo-European
		South Indians	Dravidian
	Nepal	Nepalese	Indo-European
		Tharu	Indo-European
Central/East/ Southeast Asia	Uzbekistan	Uzbeks	Altaic
	Mongolia	Khalka	Altaic
		Mongols	Altaic
	Russia	Northern Yakuts	Altaic
		Yakuts	Altaic
	Singapore	Han-Chinese	Sino-Tibetan
		Japanese	Japonic

2.1.2 African samples

Information about the African and Middle Eastern samples used in chapters 5 and 6 were extracted from the theses of Bryony Jones and Kate Ingram where they were studied in detail (Ingram 2008; Jones 2012). Table 2.2 summarises anthropological information such as broad geographic location, language families and lifestyle patterns of these populations.

Table 2.2: Summary of geography, language family and subsistence strategy of the African and Middle Eastern populations used for comparative analyses (compiled from Ingram 2008; Jones 2012).

Populations	Country	Language family	Settlement pattern	Primary subsistence method	Presence of milking	Predominant milked animal
Afar	Ethiopia	Afro-Asiatic	Nomadic	Pastoralism	Yes	Camels
Amhara	Ethiopia	Afro-Asiatic	Sedentary	Farming	Yes	Cattle
Asante	Ghana	Niger-Congo	Sedentary	Farming	No	-
Beni Amer	Sudan	Afro-Asiatic	Nomadic	Pastoralism	Yes	Camels
Chagga	Tanzania	Niger-Congo	Sedentary	Farming	Yes	Cattle
Chewa	Malawi	Niger-Congo	Sedentary	Farming	No	-
Jaali	Sudan	Afro-Asiatic	Sedentary	Pastoralism	Yes	Cattle
Mambila	Cameroon	Niger-Congo	Sedentary	Farming	No	-
Oromo	Ethiopia	Afro-Asiatic	Nomadic	Pastoralism	Yes	Cattle
Shabo	Ethiopia	Nilo-Saharan	Sedentary	Farming, hunting, gathering	No	-
Bedouin	Israel, Jordan, Saudi Arabia	Afro-Asiatic	Nomadic	Pastoralism	Yes	Camels
Israeli Arabs, Palestinians	Israel	Afro-Asiatic	Sedentary	Farming	Yes	Cattle

2.2 Experimental methods

2.2.1 DNA collection

DNA samples newly collected during the course of this thesis were obtained from buccal cell DNA. For this collection, cotton swabs were rubbed around on the inside of the cheek of the sample donors for at least 30 seconds and 10 of the swabs were placed in a 15ml Falcon tube containing 2.5ml 'Slagboom' buffer including proteinase K (see 2.5 for recipe). The tubes were stored in a dark environment at room temperature until extraction.

2.2.2 DNA extraction

The samples used in this thesis were extracted by a variety of different procedures, depending on the cellular source of the DNA and the laboratory from which they were obtained by different adaptations of the phenol-chloroform method or salting out precipitation methods.

2.2.2.1 Within group extraction method

The buccal cell samples newly collected for this thesis and other buccal samples prepared within the Swallow group were extracted by Ranji Araseretnam or Kate Ingram following an extraction protocol devised by Freeman et al. (2003).

Each collection tube containing Slagboom buffer and cotton swabs was incubated at 65°C for 2 hours. After removing the cap, the tube was inverted into a clean 50ml tube and both centrifuged for 10 minutes at 650x g. The original tube was discarded, the swabs were drained and all liquid was transferred into a new 15ml tube. 300µl Yeast Reagent 3 (Autogen Bioclear, diluted 1:1 with 100% ethanol) was added. This contains potassium acetate and phenol and chloroform to denature remaining proteins. The solution was vigorously mixed manually for 1 minute and then centrifuged for 25 minutes at 8000x g. A repeat of this step followed, after transfer of the supernatant to clean tube and further addition of 300µl Yeast Reagent 3. The supernatant containing the DNA was transferred again into a clean tube. 1.8ml 100% isopropanol was added and the tube gently inverted to mix, and precipitate the DNA. A centrifugation step followed at 8000x g for 25 minutes. The supernatant was discarded and the DNA containing pellet washed gently with 1ml 70% ethanol and centrifuged again at 8000x g for 10 minutes. The ethanol supernatant was then discarded and the tube left open to allow the pellet to air dry for 15 minutes. The DNA pellet was resuspended in 400µl Puregene DNA hydration solution (1mM EDTA,

10mM Tris-Cl (pH 7.5), Gentra) and incubated overnight at 4°C on a rocking platform. Sample aliquots were stored at +4°C and -70°C.

DNA from blood and cell line samples was extracted previously using Puregene DNA extraction kits, which involve salting out precipitation, following the manufacturer's instructions.

2.2.2.2 Extraction from dried blood

The DNA of 43 phenotyped Yakut samples was extracted from blood dried onto filter paper, using the QIAamp DNA Mini Kit (QIAGEN). The procedure followed the protocol 'DNA purification from dried blood spots':

Approximately 1cm² of paper was cut from each blood spot with scissors, cut into 4 smaller pieces and placed in a 1.5ml eppendorf tube. After each sample the scissors and forceps used were washed with 1% Alconox solution (Sigma-Aldrich), rinsed with dH₂O and 70% ethanol and dried with a separate tissue to avoid cross contamination of the samples. 180µl of Buffer ATL was added and the tube incubated at 85°C for 10 minutes. The addition of 20µl of proteinase K stock solution (600 mU/ml) followed, the mix was vortexed briefly and incubated at 56°C for 1 hour. 200µl lysis buffer (Buffer AL) was added to the sample, the solution mixed thoroughly by vortexing and incubated at 70°C for 10 minutes. 200µl 100% ethanol was added, the mix quickly vortexed and then transferred to a QIAamp mini spin column in a 2ml collection tube.

After centrifuging at 6000x g for 1 minute, the tube with supernatant containing proteins and other contaminants was discarded and the column transferred into a new collection tube. The DNA absorbed onto the silica membrane of the column was then washed in two steps to remove any remaining contaminants. First 500µl Buffer AW1 (96% ethanol) was added to the spin column and it was centrifuged at 6000x g for 1 minute. The collection tube with the filtrate was emptied and the second washing step followed with the addition of 500µl Buffer AW2 (96% ethanol) and centrifugation at 16000x g for 3 minutes. The spin column was placed in a 1.5ml eppendorf tube and 150µl Buffer AE (10mM Tris Cl, 0.5mM EDTA, pH 9.0) was added to the column and incubated for 1 minute. In the following centrifugation step at 6000x g for 1 minute the DNA was eluted from the membrane. The samples were stored at -20°C.

2.2.3 Lactase persistence data and lactose tolerance testing

As described in section 1.2.4 of the introduction, the classification of the lactase persistence status of an individual can be made from biopsy material in measuring lactase activity directly or indirectly via lactose tolerance tests. Most of the phenotype data used

in chapters 5 and 6 were obtained during the course of several other projects and were reassessed for this thesis. Test methods are briefly mentioned below, further details can be obtained from the corresponding publications.

The Northern European sample series was first analysed by Harvey et al. (1995b).

Duodenal biopsy material from hospital patients (UCLH) was examined for lactase and sucrase enzyme activity, and checked by histology.

Phenotypes of the Italian samples were also determined from lactase activity of biopsy samples, collected from hospital patients in Naples who underwent jejunal surgery (Harvey et al. 1998; Maiuri et al. 1991).

The Finnish samples were lactose tolerance tested by Dr Riitta Korpela in Helsinki via urinary galactose and breath-hydrogen tests. This cohort does not represent a randomly chosen population sample as it was collected to contain approximately half lactase persistent and non-persistent female individuals inferred from lactose digester/non-digester status (Harvey et al. 1998; Hollox 2000), which is higher than the population average for lactase non-persistence and was therefore only used for analyses in chapter 6. Lactose tolerance testing of the Yakut samples was done via blood glucose tests, conducted by Sardana Markova and Maria Khandy of the Yakutsk State University (Hollox 2000).

The volunteers from the African groups were tested by the breath hydrogen method and this was done by Mohamed Elamin from Khartoum, Tamiru Oljira from Addis Ababa University and Kate Ingram from UCL: The Jaali were from Sudan (Ingram et al. 2007; Ingram et al. 2009b) and the Somali camel herders, the Amhara and the Oromo groups were from Ethiopia (Jones 2012).

5 individuals from Northern Europe were also newly tested with this method, which can be briefly described as follows. The volunteers were given a lactose load of 50g dissolved in water after fasting overnight. Breath hydrogen was measured before and every 30 minutes (up to 3 hours) after drinking the lactose solution, with a MicroH2 meter (Micro Medical) (Peuhkuri et al. 1998). Volunteers were classified as lactose non-digesters if they showed and maintained an increase in breath hydrogen of at least 20ppm above the baseline, and as digesters with values below this. 2 individuals with a baseline and all test results lower than 2ppm were excluded as they could have been potential hydrogen non-producers.

2.2.4 Sequencing

All genomic regions of interest were firstly amplified via polymerase chain reaction (PCR) to be later analysed by sequencing: The *LCT* enhancer region in intron 13 of *MCM6*; for haplotype analysis a control region in intron 4 of *MCM6* and 3 sequence regions containing the *LCT* core haplotype defining SNPs (hapdef) previously described by Hollox et al. (2001), and the immediate promoter (IP) and parts of intron 2 of *LCT* (Figure 2.1).

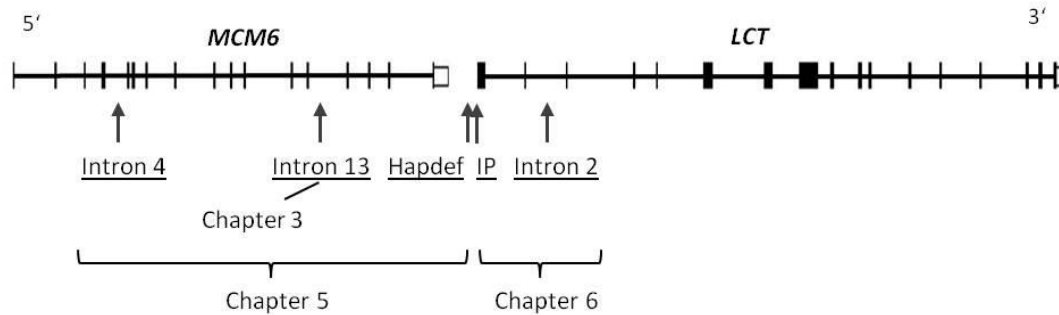


Figure 2.1: Overview about the sequenced regions for the different chapters of this thesis.

Some of the data collection was part of undergraduate projects. Most of the data for the *LCT* enhancer and immediate promoter region for South and East Asia (India, China and Japan) was collected by Anshua Gosh (Ghosh 2010). Some of the haplotype marker data for the Middle Eastern populations of Kuwait, Iran and Syria was accumulated by Lana-Mai Couzens (Couzens 2011).

The sequences of intron 4 of *MCM6*, the lactase enhancer and hapdef regions for the African populations were obtained from previous projects of Kate Ingram, Pawel Zmarz and Bryony Jones (Ingram 2008; Ingram et al. 2009b; Jones 2012; Zmarz 2010). Kate Ingram also performed the sequencing of the *LCT* enhancer and the genotyping of some haplotype defining SNPs for the Bedouin, Israeli and Palestinian populations and the European phenotyped samples (Ingram et al. 2007).

All of these sequences were re-checked and re-sequenced if necessary for chapters 5 and 6 of this project and all remaining samples were tested as part of this project.

2.2.4.1 Primer design

Primers for a multiplex PCR were designed to simultaneously amplify two of the haplotype marker regions of *LCT* exon 2/intron 1 and exon 17 of the haplotype defining regions. The sequences for the two regions were obtained from the human reference sequence, downloaded from the Ensembl database (build 71, GRCh37/hg19) and the FastPCR

software (version 5.4.8, Kalendar et al. 2011) was used for primer design. Primer specificity was checked with the 'BLAT search' of the UCSC browser (Kent et al. 2002). The primers for *LCT* intron 2 were also designed during the course of this PhD, by Bryony Jones.

2.2.4.2 PCR

Sampled DNA was PCR amplified using conditions optimized for each genomic region (temperature and cycling conditions). The multiplex PCR needed additional adjustment for optimal dNTP and MgCl₂ content. Primer sequences and reaction specific PCR cycling conditions are summarized in Table 2.3.

PCR reactions were carried out in 96 well plates each in a total volume of 10µl. Standard reactions contained 1x Reaction Buffer IV, 0.15mM MgCl₂ (0.25mM for Multiplex), 200µM dNTPs (400µM for Multiplex), 0.25U *Taq* DNA polymerase (all Thermo Fisher), 0.25-0.5µM of each primer (Sigma-Aldrich) and about 10-20ng genomic DNA. The basic method included an initial denaturation step at 95°C for 5 minutes, followed by a variable number of cycles of denaturation (95°C for 30sec), annealing (variable temperature for 30sec) and elongation (72°C for a variable time). A final elongation step followed for 5 minutes at 72°C.

2.2.4.3 Gel electrophoresis

All PCR products (after the PCR and after the PCR cleanup) were separated on 1 or 2% agarose gels in comparison with a DNA ladder (Stretch marker, see 2.5.1), to check their quantity and ensure the correct sizes. 2µl of the PCR product mixed with 3µl loading buffer was loaded on the gels that were stained with 50ng/ml ethidium bromide (Sigma) that was included in the gel. The gels were visualised and photographed under UV light for documentation.

2.2.4.4 PCR cleanup

To prepare the PCR products for the sequencing process they needed to be purified. The PCR cleanup protocol is essentially a polyethylene glycol (PEG) precipitation method: The precipitation solution (2/3 'Microclean', see section 2.5.1) was added to the PCR product in 3 fold volume. The plates were mixed thoroughly and centrifuged at 1500x g for 1 hour. The supernatant, containing primers, excess nucleotides and salts was then discarded by inverting the plate onto tissue and centrifuging at 20x g for 1 minute. A washing step followed to remove the remaining PEG and any contaminants with the addition of 150µl

70% ethanol, centrifugation for 10 minutes at 1500x g. The ethanol solution was discarded as before by inverting and centrifuging of the plate at very low speed (20x g) for 30 seconds. The DNA pellets were left at room temperature to air dry for at least 15 minutes and were then resuspended in 10µl dH₂O.

2.2.4.5 Sequencing

All sequencing reactions were performed by myself or carried out by Mari-Wyn Burley from 'The Centre for Comparative Genomics' at UCL using a standard protocol based on the 'Sanger sequencing' method (Sanger et al. 1977) and the BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, ABI). For the reactions either PCR primers or additionally designed sequencing primers were used (Table 2.3).

Each sequencing reaction was carried out in a 10µl volume with 2-2.5µl cleaned up PCR product. The mix further contained 0.35µl BigDye Terminator v3.1, 1x sequencing buffer and 0.16µM primer. Thermo cycle sequencing was performed using the following parameters: After an initial denaturation at 96°C for 1 minute 25 cycles of 96°C for 1 minute, 50°C for 5 seconds and 60°C for 4 minutes followed.

Following the sequencing reaction, the DNA was purified by ethanol precipitation to remove contaminants. 2.5µl of 125mM EDTA and then 30µl 100% ethanol was added to each well. The plate was thoroughly mixed and centrifuged for 1 hour at 3870rpm. The supernatant was removed by inverting the plate onto tissue and centrifuging at 300rpm for a few seconds. Any remaining contaminants were then removed by the final ethanol wash: 30µl of 70% ethanol was added to each well and the plate centrifuged again at 3870rpm for 10 minutes. The ethanol was removed by inverting the plate onto tissue again and centrifugation at 300rpm for a few seconds. The plate with the DNA pellets was left at room temperature to air dry for 15 minutes.

To prepare the samples for the run on an ABI 3730xl DNA Analyzer, the pellets were resuspended in 10µl HiDi formamide (ABI), denatured at 95°C for 5 minutes and then immediately put on ice for at least 5 minutes before they were loaded in the machine.

2.2.4.6 Sequence analysis

Sequence chromatograms were analysed using the software ChromasPro (version 1.5, Technelysium Pty Ltd) and compared to a reference sequence (Ensembl database, GRCh37/hg19). The DNA was mostly sequenced in both directions. New variants were in

each case confirmed on both strands or sequencing of at least one additional and independent PCR.

The ancestral allelic state at each new locus was inferred by comparing the human reference sequence for each genetic region with those of other primate species (see Appendix A). The human reference sequence (Feb 2009, GRCh37/hg19) for each region was downloaded from the UCSC browser (Kent et al. 2002) using the flanking PCR primers for the 'In-Silico PCR' tool. The corresponding primate sequences were generated using the UCSC 'BLAT search' with the reference sequence assemblies for chimpanzee (Feb 2011, CGSC 2.1.4/panTro4), orang-utan (July 2007, WUGSC 2.0.2/ponAbe2), gorilla (May 2011, gorGor3.1/GorGor3) and rhesus macaque (Oct 2010, BGI CR_1.0/rheMac3). The BioEdit sequence alignment editor (version 7.0.9.0, Hall 1999) and the integrated CLUSTAL W (version 1.81) multiple sequence alignment tool (Thompson et al. 1994) was used to align the sequences.

2.2.5 Genotyping

2.2.5.1 KASP genotyping

Genotyping of 880 samples for analysis in chapter 5 was performed externally by LGC Genomics (KBioscience) using their KASP ('Kompetitive allele specific PCR') genotyping assay. Results were viewed with SNPviewer2 (version 3.2.2.16), provided by the company. Quality control checks were performed after the data were provided. For each population, the SNP calls were visually inspected to check they were assigned to the correct genotype. Uncertain calls (marked as '?') that could be clearly grouped with either one of the homozygous or the heterozygous genotypes were corrected manually. Finally the data were tested as to whether they agree with Hardy Weinberg expectations (as described in section 2.3.1).

Table 2.3: Primers used for PCR amplification and sequencing at UCL

Genetic region	Location (bp 5' of <i>LCT</i> start of transcription)	Primer name	Primer sequence (5'-3')	Product length	Annealing temperature (°C)	Elongation time (sec)	cycles
<i>LCT</i> enhancer <i>MCM6</i> , intron 13	-14,397	MCM6ex13 MCM6778	ATTTCCAAAGAGTCAGAGGACTTC CCTGTGGGATAAAAGTAGTGATTG	940	58	60	39
	-14,163	MCM6i13 MCM6778	GGACATACTAGAATTCAGTCAAATAC CCTGTGGGATAAAAGTAGTGATTG	706	58	45	38
		MCM6ex13 LAC-C-L2	ATTTCCAAAGAGTCAGAGGACTTC CTGCTTTGGTTGAAGCGAAGAT	661	58	45	38
<i>LCT</i> , immediate promoter	-527	LCT IP_f LCT IP_r	TCTTCAGACATTTTCCGGGTTC TGTCTTTGTCCCCTGCTACA	719	56	45	37
<i>LCT</i> haplotype marker <i>LCT</i> , hapdef region	-1,162	LCT_far_prom_f LCT_far_prom_r	ATCCACATTCTACAGGTGACAA GACCAACACAAAAACCTCAGAC	701	59	60	38
<i>LCT</i> , exon 2	3,762	LCT_Ex2_F LCT_Ex2_R	CCTTGCCAACTCTCCAACTGC ACACTTGACCAAGCAGGAGC	502	59	45	38
<i>LCT</i> , exon 17	48,500	LCT_Ex17_F LCT_Ex17_R	GATGGGTACCTCCACCTCG TGGTGAGAAAGCTTAATCGGAGC	927			
<i>LCT</i> , intron 2	5,282	LCT_intron2_f LCT_intron2_r	CTGGGAAGTGAACAGCTTTGG CCACCACATTAAGTCTTGGTCTCC	839	58	60	39
<i>MCM6</i> , intron 4	30,461	MCM6_intron4f MCM6_intron4r	ACCCTCAGATTTTCAGCAGGAC ACTCCATGATGATTCAAGCAGC	683	58	45	39

Other sequencing primers	Primer name	Primer sequence (5'-3')
<i>LCT</i> , inner promoter	LCT IP	AGATGGAGTCTCATTCTGTTGC
<i>LCT</i> , hapdef region	LCT-farprom_hap_seq_primer(r)	TTGGTGACCGGGTCTCACTCTG
<i>MCM6</i> , intron 4	intron4_R2	GACTCCAAATATCTGTTCCATGC

2.2.6 Electrophoretic mobility shift assays (EMSAs)

EMSAs were performed to detect nuclear proteins in the Caco-2 cell line that bind to DNA sequences of the *LCT* enhancer and distinguish possible differences in binding affinity to probes containing the derived or ancestral allelic variants.

2.2.6.1 Cell culture

Human Caco-2 cells (obtained from Martin Spiess, Basel, passage >80) were cultured in 180ml tissue culture flasks and kept in a humid environment at 37°C and 5% CO₂. The cells were grown in 30ml Dulbecco's modified eagle medium (DMEM, with 4.5 g/L Glucose and L-Glutamine, BioWhittaker-Lonza), supplemented by 100U/ml penicillin, 100µg/ml streptomycin and 10% foetal calf serum (Remal, all Fisher Scientific). At 80% confluence (after 3-4 days) the cells were split by trypsinisation. To do this the medium was discarded from the Caco-2 monolayer culture and the cells were washed with 5ml 0.05% Trypsin-EDTA solution (Gibco, Life Technologies) which was then all discarded with exception of a small amount just covering the cells. A short incubation followed for up to 5 minutes at 37°C until the cells could be detached from the flask surface by tapping the flask. 10ml of fresh DMEM 10% FCS, (full culture medium) were added (using the anti-trypsin in the FCS) to stop the reaction and suspend the cells. To start a new passage of cell culture 1ml of DMEM-containing cell solution was transferred to a new culture flask and 29ml culture medium added. The medium was changed at 2 day intervals.

2.2.6.2 Preparation of nuclear protein extract

Caco-2 cells were grown on 20 x 20cm cell culture plates. After 10 days of culture cells had grown to confluence and the formation of domes was visible by inspection under a microscope as described by Pinto and colleagues (Pinto et al. 1983b) indicating that they had become differentiated.

Nuclear protein extract was prepared from differentiated Caco-2 cells 13 days after seeding following the protocol described in Ausubel (2002). The medium was removed from the cells, they were washed twice in phosphate-buffered saline (PBS, pH 7.4, Gibco, Life technologies) and harvested by scraping them off the surface in 40ml PBS. A centrifugation step followed at 3000rpm for 10 minutes and the supernatant was discarded. The cell pellet was immediately resuspended in hypotonic buffer (see 2.5) of 5x the volume of the cell pellet and centrifuged for 5 minutes at 3000rpm to remove salt from the PBS solution for effective swelling in the next step. The supernatant was discarded and the cell pellet resuspended again in hypotonic buffer to a final volume of 3x the first pellet

volume and allowed to swell on ice for 10 minutes. In the following step the cells were homogenized in a glass Dounce homogenizer by 10 very slow up-and-down strokes with a type B pestle to lyse the cells and release the nuclei. Cell nuclei were collected by centrifuging at 3300x g for 15 minutes and after that resuspended in low-salt buffer of half the volume of the nuclear pellet. This was followed by application of the same volume (half the original nuclear pellet) of high-salt buffer (for recipes see 2.5.1) dropwise with continuous and gentle stirring and the nuclei were allowed to extract under further gentle mixing for 30 minutes. The lysed nuclei were pelleted by centrifugation at 20000x g for 30 minutes and the nuclear extract in form of the supernatant placed in dialysis tubing (Visking dialysis membrane, diameter size 6.3mm, volume 0.3ml/cm, molecular weight cut-off 12-14 kDa, Medicell) and left to dialyse against 50 volumes of dialysis buffer overnight. The extract of the dialysis bag was then centrifuged at 14500rpm for 20 minutes and the clean extract between the pellet and precipitates on the supernatant surface was removed, aliquoted and stored at -80°C.

The protein concentration of the nuclear extract was measured by the method of Bradford (1976) using the 'Bio-Rad Protein Assay' microassay procedure. 5 dilutions of the protein standard (Protein standard II, bovine serum albumin, Bio-Rad), to create a standard curve, and 2 dilutions of the nuclear extract with PBS in a 10µl volume were made in duplicate. 10µl of dialysis buffer was added and the mixture made up to 800µl with dH₂O. 200µl of dye (protein assay dye reagent concentrate, Bio-Rad) was added to each tube and after vortexing the tubes were incubated at room temperature for 5 minutes. The absorbance was measured at 595nm in a photometer and the protein concentration of the extract calculated from the standard curve.

2.2.6.3 EMSA oligonucleotide design

Gel shift assays were performed with labelled DNA probes and nuclear extract, competition experiments with the addition of unlabelled short DNA sequences and supershift experiments with the addition of antibodies specific for a certain transcription factor (see also chapter 4).

Two complementary oligonucleotides for each of the five enhancer variants tested (-14028*C, -14011*T, -14009*G, -13779*C and 14010*C as control) and their corresponding ancestral sequences were designed by visual inspection of the sequence from the human reference sequence template (GRCh37/hg19). They were designed to be between 22 and 31 bp in length with the ancestral or mutated base near the centre and an extra 5' base on

both the sense and antisense oligonucleotide to create an overhang during later annealing, which is important for the labelling of the probe.

For EMSA competition experiments oligonucleotides with known transcription factor affinity sites were used, of which some had already been designed and tested by other lab members. Others were newly designed after bioinformatic analysis of the regions around the five enhancer variants (see section below and chapter 4). The core sequences consisting of matrices for predicted transcription factor binding sites were complemented with random bases at both sides to reach an oligonucleotide length of 25 bp. The oligonucleotide sequences were re-checked bioinformatically for binding specificity and the core flanking sequences modified accordingly. A 5'A base was added to both complementary oligonucleotides in case they would later be needed as probes. All double stranded oligonucleotide sequences used in EMSA experiments can be found in Table 2.4. A further set of oligonucleotides was ordered with sequences taken from technical information sheets of specific transcription factor probes from commercially available EMSA kits (Panomics, Affymetrix transcription factor EMSA kits, <http://www.affymetrix.com/estore/>).

2.2.6.4 Probe preparation: Radioactive labelling and purification

Sense and antisense oligonucleotides (MWG Operon) were annealed and used as unlabelled (cold) probe for competition experiments or labelled with radioactivity.

Annealing reactions were carried out in a volume of 100µl containing 250pmol of each primer and 0.1M NaCl. After heating the mix at 95°C for 1 minute, the tube was left in the heating block on the bench to slowly cool down to room temperature.

2.5pmol of the double stranded probes were labelled using 5U T4 Polynucleotide Kinase (Fermentas) and 25-35µCi [$\gamma^{32}\text{P}$]ATP (Perkin Elmer) in Kinase reaction buffer A (forward, Fermentas). The reaction mix was incubated for 30 minutes at 37°C and 20µl 1x TE buffer were added afterwards. The probe was purified with a MicroSpin G-25 Column (Illustra, GE Healthcare) and the eluate filled up to 100µl total volume with TE buffer.

Table 2.4: Double stranded oligonucleotides used for EMSAs, variants indicated in bold.

Probe name	Sequence
Enhancer variants	
14028T (ancestral)	5'-ACGTCATAGTTTATAGAGTGCATAAA-3' 3'-GCAGTATCAAATATCTCACGTATTTTC-5'
14028C	5'-ACGTCATAGTT C ATAGAGTGCATAAA-3' 3'-GCAGTATCA A GATCTCACGTATTTTC-3'
14011C (ancestral)	5'-TAGAGTGCATAAAGACGTAAGTTACCATTTA-3' 3'-TCTCACGTATTTCTGCATTCAATGGTAAATT-5'
14011T	5'-TAGAGTGCATAAAGAT T GTAAGTTACCATTTA-3' 3'-TCTCACGTATTTCT A CATTCAATGGTAAATT-5'
14010C	5'-TAGAGTGCATAAAGAC C TAAGTTACCATTTA-3' 3'-TCTCACGTATTTCTG G ATTCAATGGTAAATT-5'
14009G	5'-TAGAGTGCATAAAGACG G AAGTTACCATTTA-3' 3'-TCTCACGTATTTCTGC C TTCAATGGTAAATT-5'
13779G (ancestral)	5'-AGTAGTACGAAAGGGCATTCAA-3' 3'-CATCATGCTTTCCCGTAAGTTC-5'
13779C	5'-AGTAGTAC C AAAGGGCATTCAA-3' 3'-CATCATG G TTTCCCGTAAGTTC-5'
TF binding competitors	
SIF1 24 (Cdx-2 binding site)	5'-TGGGTGCAATAAACTTTATGAGTA-3' 3'-CCCACGTTATTTTGAAATACTCATT-5'
Oct control	5'-ATGTGCAATGCAAATCACTAGAA-3' 3'-ACAGCTTACGTTTAGTGATCTTA-5'
LPH-CE2c (HNF-1α binding site)	5'-ATAACCCAGTTAAATATTAAGTCTTAAT-3' 3'-TATTGGGTCAATTTATAATTCAGAATTA-5'
TREH's HNF4 site (HNF-4α binding site)	5'-CCTCAAAGGCTGGACTTTGGCCGACTTGG-3' 3'-CTCAAAGGCTGGACTTTGGCCGACTTGG-5'
mut XbaI 13910 site (2 GATA binding sites)	5'-TACAGATAAGATAATTCTAGACCTGGCCTCAAAGGA-3' 3'-ATGTCTATTCTATTAAGATCTGGACCGGAGTTTCCT-5'
unspec24 (non-specific)	5'-AACGTAGCTGATCGAATCGGTAC-3' 3'-TGCATCGACTAGCTTAGCCAATGA-5'
Newly designed TF binding competitors (see also Table 4.1)	
c-Ets-1	5'-AATCCTCTACCGGATGTAGGTCGAC-3' 3'-TAGGAGATGGCCTACATCCAGCTGA-5'
Ets/Tel-2	5'-ATCTTACTACTTCCTCGCTGACCGT-3' 3'-AGAATGATGAAGGAGCGACTGGCAA-5'
NF-kappaB	5'-AGTGGCGGGGAAAGTCCCCAGAATC-3' 3'-CACCGCCCCTTTCAGGGGTCTTAGA-5'
Pax	5'-AAGAAGTGGAACCTACGATCGTGCT-3' 3'-TCTTCACCTTGAGTGCTAGCACGAA-5'
Pax4-8	5'-ACGGCGTTCATGCGTGAGCGACCGT-3' 3'-GCCGCAAGTACGCACCTCGCTGGCAA-5'
Pbx	5'-AGATGGGATTGATGGTAGCCGTATT-3' 3'-CTACCCTAACTACCATCGGCATAAA-5'

Probe name	Sequence
Competitors from Affymetrix EMSA kit	
Aff_c-Ets-1	5'-ACCAGGAAGCCAGGAAG-3' 3'-GGTCCTTCGGTCCTTCA-5'
Aff_ETS (1)	5'-AGGAGGAGGGCTGCTTGAGGAAGTATAAGAAT-3' 3'-CCTCCTCCCGACGAACCTTCATATTCTTAA-5'
Aff_ELF	5'-AGAGTCATCAGAAGAGGAAAAATGAAGGT-3' 3'-CTCAGTAGTCTTCTCCTTTTTACTTCCAA-5'
Aff_Elk-1	5'-ATTTGCAAAATGCAGGAATTGTTTTACAGT-3' 3'-AAACGTTTTTACGTCCTTAACAAAAGTGTCAA-5'
Aff_LEF1	5'-ACCCATTTCCATGACGTCATGGTTA-3' 3'-GGGTAAAGGTACTGCAGTACCAATA-5'
Aff_GKLF	5'-AATGCAGGAGGAGAAAGAAGGGCGTAGTATCTACTAG-3' 3'-TACGTCCTCCTCTTTCTTCCCGCATCATAGATGATCA-5'
Aff_AML1	5'-AATCTCTATGTGGGTGTGGGTGTGGGAATCAT-3' 3'-TAGAGATACACCCACACCCACACCCTTAGTAA-5'
Aff_GATA3	5'-AGTTATTTATCTCTTAGTTGTAGTTATTTATCTCTTAGTTGTA-3' 3'-CAATAAATAGAGAATCAACATCAATAAATAGAGAATCAACATA-5'
Aff_GATA4	5'-AGCCTAAGCCAAGTGATAAGCAGCCAGACAA-3' 3'-CGGATTTCGGTTCCTATTCGTCGGTCTGTTA-5'

2.2.6.5 Protein binding reaction

A reaction mix, containing both the extract and radioactive end-labelled DNA fragments was prepared. Binding reactions for EMSA experiments were usually performed with 4.5-9µg differentiated Caco-2 nuclear extract, 4µl dialysis buffer, 10µl gelshift buffer. Each reaction also contained 0.25µg Poly (deoxyinosinic-deoxycytidylic) acid sodium salt (poly dI-dC, Sigma-Aldrich) and 2.5 pmol unlabelled oligonucleotides to prevent nonspecific DNA binding for clearer bands on the gel pictures.

Most reactions showed both specific and non-specific binding proteins. To specify further which protein of the extract is binding, competition and supershift experiments were performed. For competition assays, 2.5pmol of the unlabeled annealed competitor oligonucleotide was added to the reaction mix and for supershift assays 1-2µl of the antibodies Cdx-2 (BioGenex), Oct-1, HNF-1α, HNF-4α, Ets-1/2, Ets-1, GATA-6 (all Santa Cruz).

After incubation of the mixture for 10 minutes on ice 2.5 fmol of probe was added, mixed and incubated on ice for at least further 20 minutes.

2.2.6.6 Polyacrylamide (PAA) gel electrophoresis and analysis

For electrophoresis, 5% nondenaturing polyacrylamide gels (30% acrylamide:bisacrylamide 29:1, Sigma-Aldrich) were prepared. The PAA-gel was pre-run at 4°C for 1 hour using 0.5x TBE buffer before the mixes of the binding reactions, resolved

in 2µl EMSA loading buffer, were loaded and run at 4°C. Following electrophoresis the gel was transferred onto Whatman paper and vacuum dried for at least 2 hours at about 80°C. The gel-paper was exposed to a phosphoimaging screen for at least 2 hours which was then scanned with a Storm scanner and analysed using the Imagequant software (version 5.1, GE Healthcare).

2.2.7 Transfection studies

Transfection studies were performed to examine the effect of the different *LCT* enhancer and promoter variants, introduced in a reporter gene construct, on the expression of the reporter gene luciferase. Figure 2.2 summarises the cloning strategies described below.

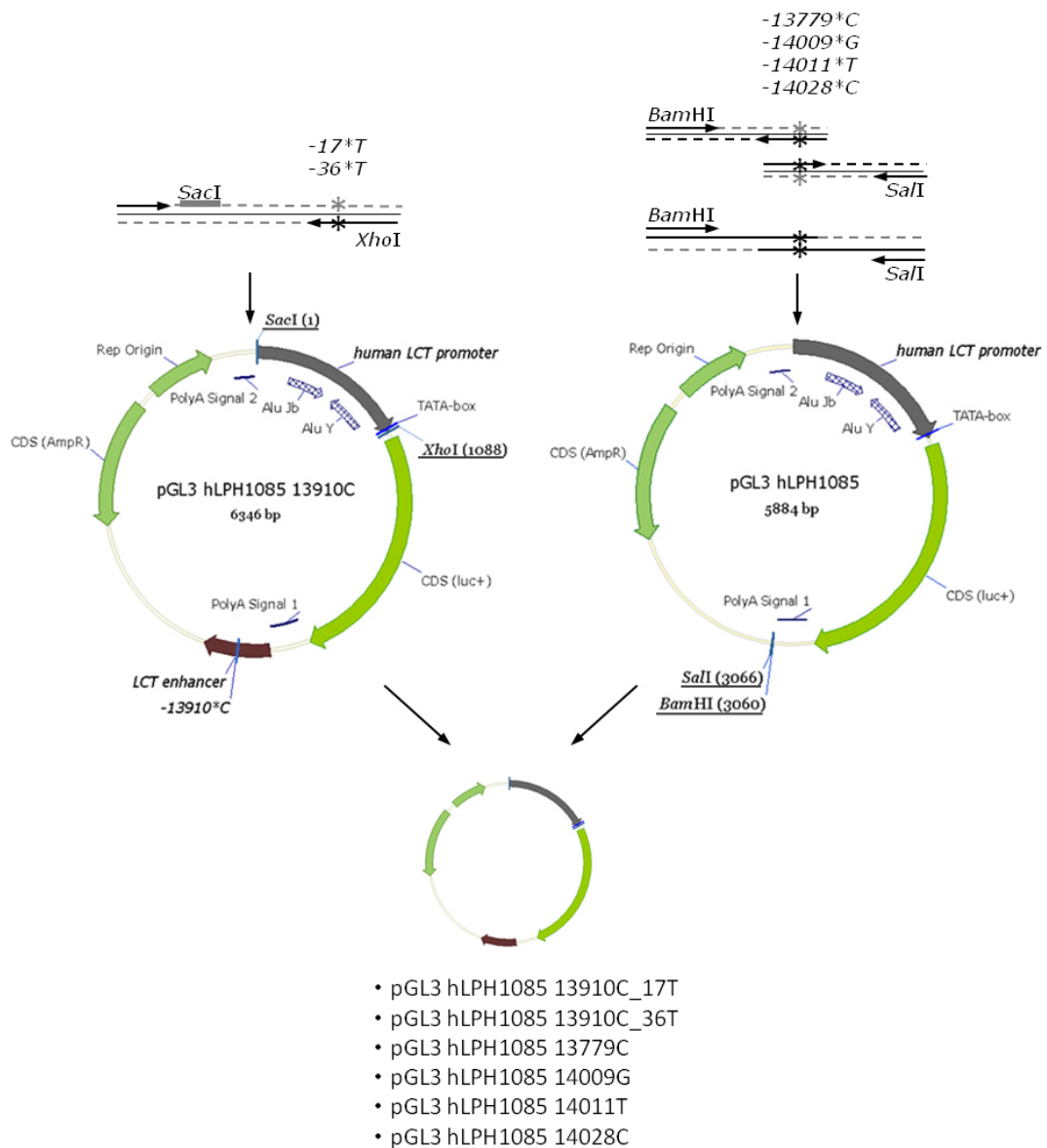


Figure 2.2: Summary of the different strategies for site directed mutagenesis and insertion of *LCT* promoter fragments (left) and enhancer fragments (right) into the luciferase reporter gene plasmids. *LCT* promoter variants were created in one PCR step and inserted via *SacI* and *XhoI* into a plasmid, containing the ancestral enhancer region (pGL3 hLPH1085-13910*C (Troelsen et al. 2003)). *LCT* enhancer variants were generated in a two step PCR amplification and inserted via *BamHI* and *SalI* into the pGL3vector containing the ancestral promoter region (pGL3 hLPH1085 (Troelsen et al. 2003)). Vector maps were created using Vector NTI Advance software (version 11.5, Invitrogen).

2.2.7.1 Preparation of the *LCT* enhancer fragments

The *LCT* enhancer variants were generated via site directed mutagenesis in a two step PCR amplification of the pGL3 hLPH1085-13910C enhancer plasmid construct (Troelsen et al. 2003). Primers designed to add an upstream 5' *BamHI* and downstream 3' *SalI* restriction

site to the 450 bp enhancer fragment were used together with the overlapping primers designed for EMSA experiments to insert the variants (see Table 2.5).

First, the region was amplified in two separate PCRs to generate a left and right fragment (with the *Bam*HI site including primer and the reverse variant EMSA primer for the left fragment and the primer including the *Sal*I site and the forward variant EMSA primer for the right fragment). PCR reactions contained 1x *Pfu* Buffer with MgSO₄, 200μM dNTPs, 2.5U recombinant *Pfu* DNA polymerase, all Fermentas), 0.4μM of each primer (MWG Operon) and about 37ng of the pGL3 hLPH1085-13910*C plasmid as template. Cycling conditions are described below (2.2.7.3).

The two fragments, in form of 1μl of the purified left and right fragment, were then annealed to create an overlap at the mutated position with a denaturation step at 94°C for 1 minute and an annealing step at 72°C for 3 minutes prior the second PCR (for cycling conditions see 2.2.7.3). The flanking *Bam*HI and *Sal*I site containing primers were then used to amplify the whole enhancer fragment.

2.2.7.2 Preparation of the LCT promoter fragments

The *LCT* promoter variants were created in one PCR step with the mutations directly included in the 3' primers, also with the pGL3 hLPH1085-13910C plasmid as template. Primers containing the -36*T and -17*T variants and a 3' *Xho*I restriction site were designed and used together with a previous designed 5' primer to create a fragment containing a *Sac*I restriction site via PCR.

Table 2.5: Primers used for PCRs to create fragments to be inserted into reporter gene plasmids (mutated bases are indicated in bold).

Genetic region	Primer name	Primer sequence (5'-3')
LCT enhancer	14028C_Fwd	ACGTCATAGTTCATAGAGTGCATAAA
	14028C_Rev	CTTTATGCACTCTAT G AACTATGACG
	14011T_Fwd	TAGAGTGCATAAAGATGTAAGTTACCATTTA
	14011T_Rev	TTAAATGGTAACTTAC A CTTTATGCACTCT
	14009G_Fwd	TATAGTGCATAAAGACG G AAGTTACCATTTA
	14009G_Rev	TTAAATGGTAACTT C CGTCTTTATGCACTCT
	13779C_Fwd	AGTAGTAC C AAAGGGCATTCAA
	13779C_Rev	CTTGAATGCCCTTT G GTACTAC
	pcr14_5_131bp+ (BamHI)	GGATCCTTTATGTA A CTGTTGAATGC
	pcr14_3_149bp- (Sall)	GTCGACTTTCAAAGACGACCTTACAT
LCT promoter	5 pGL3 primer 3	CTAGCAAAATAGGCTGTCCC
	LPH_pro_36C>T_rev	CTCGAGATGTGGAACCCCTTACTTTATACT A CAACT
	LPH_pro_17C>T_rev	CTCGAGATGTG A AACCCCTTACTTTATACTGC
Sequencing primer	Primer name	Primer sequence (5'-3')
	lucsal	GTCATAAGTGC G GCGACGATAGT
	3pGL3 primer 1	CTTTATGTTTTTGGCGTCTTCCA
	5 pGL3 primer 3	CTAGCAAAATAGGCTGTCCC

2.2.7.3 PCR and gel purification

The PCR reactions to amplify the promoter fragments and the full enhancer (second PCR) were performed in a 25µl volume using the Advantage-GC cDNA PCR kit (Fermentas), which also creates a 5' overhang to the amplified product, important for later cloning. The reactions contained 1x cDNA PCR reaction buffer, 1x dNTP mix (200µM), 1x Advantage-GC cDNA polymerase mix, 0.2µM of each primer (MWG Operon) and about 37ng of the pGL3 hLPH1085-13910*C plasmid (or as template instead 2µl of the annealed enhancer fragment (section 2.2.7.1) or picked bacterial clones for colony PCR (section 2.2.7.5)).

The general procedure for the PCR included an initial denaturation step at 95°C for 1 minute followed by 25 cycles of denaturation (95°C for 30sec), annealing (55°C for 30sec) and elongation (72°C for 1min for enhancer or 2 min for promoter PCR) and a final elongation step for 5 minutes at 72°C.

The PCR products were run on a 1.5% agarose gel (as described in 2.2.4.3) with a marker lane (usually GeneRuler 100 bp, 100 bp Plus or 1 kb DNA Ladder, Fermentas). Bands of the right size were cut out of the gel and purified using the NucleoSpin Extract II kit (Macherey & Nagel) and the protocol for 'DNA extraction from agarose gels'. Briefly, the agarose gel slice and 200µl Buffer NT (high salt buffer) per 100mg gel were incubated in a tube at 50°C and vortexed every 2-3 minutes until the gel piece was dissolved completely. Then the mixture was placed on a prepared NucleoSpin Column and centrifuged for 1 minute at 11000x g to bind the plasmid DNA to the silica membrane of the column. In the following washing step all contaminants were removed. 600µl Buffer NT3 (96-100% ethanol) were added and the column centrifuged at 11000x g for 1 minute, after removing of the flow-through a further centrifugation step for 2 minutes followed. The column membrane was incubated with 15µl Buffer NE (5mM Tris-HCl, pH 8.5) for 1 minute and the DNA eluted in a new tube by centrifuging at 11000x g for 1 minute.

2.2.7.4 Cloning of PCR products

All purified enhancer and promoter fragments were TA-cloned into the pCR 2.1-TOPO plasmid using the TOPO-TA Cloning kit (Invitrogen). The 3'T overhang of the active plasmid vector was ligated to the 5'A of the PCR product with the help of Topoisomerase I, bound to the vector.

4µl of the purified PCR product and 1µl Salt Solution was added to 10ng of the pCR 2.1-TOPO plasmid DNA. After mixing and incubation at room temperature for 5 min, the mixture was transferred on ice.

The transformation into One Shot TOP10 *E.coli* competent cells (genotype: F- *mcrA* Δ (*mrr-hsdRMS-mcrBC*) Φ 80*lacZ* Δ M15 Δ *lacX74* *recA1* *araD139* Δ (*araleu*) 7697 *galU* *galK* *rpsL* (Str^R) *endA1* *nupG*) was performed with 2µl of each TOPO cloning reaction added to a vial containing 25µl cells. After gentle mixing and incubation on ice for 30 minutes the cells were heat shocked for 30 seconds at 42°C to permeabilize the cell membrane for plasmid uptake, and transferred back on ice. 250µl Super Optimal broth with Catabolite Repression (S.O.C.) medium was added to the cells and the tubes placed on a shaking platform (170rpm) to incubate for 1 hour at 37°C.

Each cell solution was then divided and spread on two pre-warmed agar plates (20ml LB agar, Sigma-Aldrich), containing 50µg/ml ampicillin and 40µg/ml 5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside (X-gal, Invitrogen), and incubated at 37°C and 5% CO₂ over night. The plates were screened for blue and white colonies the next day. 5 white colonies

were picked per variant clone and incubated over night (37 °C, 5% CO₂) in 3ml LB medium (Lennox LB Broth, Sigma-Aldrich), containing 50µg/ml ampicillin.

2.2.7.5 *Plasmid preparation (Miniprep)*

1.5ml of each bacterial culture was used for a miniprep using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich) following the protocol provided. All centrifugation steps were performed at 12000x g if not stated otherwise. Bacterial cells were pelleted for 1 minute and the supernatant discarded. The cells were completely resuspended in 200µl Resuspension solution and 200µl Lysis solution was added. The tube was inverted 6-8 times to mix gently. After the mixture became clear and viscous 350µl Neutralisation solution was added and gently mixed by inverting the tube 4-6 times to precipitate cell debris, proteins, lipids, salts and chromosomal DNA. By centrifuging for 10 minutes the debris was pelleted. The cleared lysate supernatant containing the plasmid DNA was transferred to a prepared binding column and centrifuged at 16000x g for 1 minute. The flow-through was removed and the column washed with 750µl Wash solution under centrifugation for 1 minute to remove contaminants. The flow-through was discarded and the columns dried in a further 1 minute centrifugation step. The purified plasmid DNA was eluted in a new collection tube with the addition of 100µl Elution solution to the column and a centrifugation for 1 minute.

The inserts of the plasmids were checked to be the right sized fragments on agarose gels subsequent to either restriction enzyme digest within the ligation preparation step (see below) or PCR of individual colonies. Agarose gel purification followed as described in section 2.2.7.3.

2.2.7.6 *Enzyme digest, ligation and transformation of the pGL3 plasmids*

The promoter fragments were inserted into the pGL3 hLPH1085-13910*C plasmid using *SacI* and *XhoI* digestion and enhancer fragments were inserted into pGL3 hLPH1085 with *SalI* and *BamHI* digest. Both pGL3 plasmids needed to be opened prior to ligation and the mutated *LCT* enhancer and promoter fragments cut out of the plasmid via restriction enzyme digestion.

The optimal conditions for the digestion with two restriction enzymes were calculated using Fermentas' DoubleDigest web tool (June 2010, now Thermo Scientific).

Reactions with *BamHI* and *SalI* were carried out in a 20µl volume with 10µl 'miniprep' plasmid DNA containing the mutated *LCT* enhancer, 1U *BamHI* and 2U *SalI* in 1x Buffer *BamHI* (all Fermentas) or in a 40µl reaction with ~2µg pGL3 hLPH1085 plasmid DNA and

2U *Bam*HI and 4U *Sall* in 1x Buffer *Bam*HI respectively. The mixture was incubated at 37°C for at least 1 hour.

Restriction digests with *Sac*I and *Xho*I were carried out in 2 steps. First, 10µl 'miniprep' plasmid DNA containing the mutated promoter fragments was digested with 2U *Sac*I in a reaction volume of 20µl 1x Tango Buffer or ~2µg of the pGL3 hLPH1085-13910*C with 4U *Sac*I in 40µl 1x Tango Buffer. After incubation for 1 hour at 37°C 1U *Xho*I was added to the promoter plasmid digest reaction and 2U *Xho*I to the pGL3 hLPH1085-13910*C digest. Both reaction volumes were made up with Tango buffer to a 1x concentration. A further incubation step at 37°C for at least 1 hour followed.

The digested enhancer and promoter and linearised vector fragments were separated on gels and fragments of the right size cut out of the gel and purified as described above. The 5 digestion products (from the 5 different clones) for each mutated enhancer/promoter fragment were gel purified together.

The DNA concentration of the eluate was measured using a NanoDrop Spectrophotometer and was between 10.1 and 17.5ng/µl for the enhancer and promoter fragments and 51ng/µl for pGL3 hLPH1085-13910*C and 20ng/µl for pGL3 hLPH1085 for subsequent ligation and transformation.

A sticky end ligation of enhancer/promoter fragments with the linear vector DNA was performed with the help of T4 DNA ligase (Fermentas) in a total volume of 20µl 1x T4 DNA Ligase Buffer (Fermentas). The reactions were carried out with a 5:1 ratio of the enhancer/promoter fragment DNA over the linear vector DNA with 1U T4 DNA ligase. After 10 minutes incubation at room temperature the plasmids were either used for transformation or frozen at -20°C.

5µl of the plasmid ligation mix was transformed into 40µl (1:4 diluted) XL1-Blue *E.coli* competent cells (genotype: *recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac* [F' *proAB lacI^qZAM15 Tn10* (Tetr)], Stratagene) and these spread on agar plates following the same protocol as for cloning of PCR products into TOP10 cells above (2.2.7.4).

As many as possible colonies were picked and checked for successful insertion of the right sized fragments by colony PCR (section 2.2.7.3). All clones containing inserts were chosen and grown in 3ml LB medium and 'miniprep' as above or stored in 50% glycerol cultures at -80°C. Gel purified plasmid DNA was sent out for sequencing using the Eurofins

MWG Operon sequencing service. Sequences were verified in both directions using sequencing primers flanking the inserted regions (see Table 2.5).

2.2.7.7 Plasmid preparation (Maxiprep)

One clone for each mutated plasmid, with the right sequence, was chosen and 50µl of the bacterial glycerol culture was grown in 200ml LB medium (containing 1µl/ml ampicillin) and incubated on a shaking platform (170rpm) for 16 hours (37°C, 5% CO₂). Maxipreps were performed using the NucleoBond Xtra plasmid purification kit (Macherey & Nagel) following the corresponding protocol 'High-copy plasmid purification (Midi, Maxi)'. The cells from each culture were transferred to a centrifuge flask and harvested in a centrifugation step at 6000x g and 4°C for 15 minutes. After discarding the supernatant, the cell pellet was completely re-suspended in 10ml re-suspension Buffer RES, containing RNase A to degrade RNA in the solution. 10ml lysis Buffer LYS (containing NaOH and SDS) was added to denature proteins, chromosomal and plasmid DNA. The solution was gently mixed and incubated for 5 minutes at room temperature. To precipitate salts, proteins, chromosomal DNA and put the plasmid DNA back into its supercoiled state in the solution, 10ml neutralisation Buffer NEU (containing potassium acetate) was added. Following gentle mixing, the lysate was applied to the inserted filter of the NucleoBond Xtra column, prepared with equilibration buffer (20ml Buffer EQU). The column was then washed by adding 10ml Buffer EQU to the filter rim and the filter containing unwanted contaminants discarded. The column containing silica resin was washed again with 20ml Buffer WASH and the plasmid DNA eluted from the column in 10ml elution Buffer ELU, collected in a 15ml falcon tube. The plasmid DNA was precipitated with 7ml room-temperature 100% isopropanol. The isopropanol-eluate mix was vortexed, incubated for 2 minutes and centrifuged at 15000x g for 30 minutes. The supernatant was carefully discarded and the pellet left to air dry for 10 minutes. Finally the pellet was re-dissolved in 200µl TE buffer. The DNA concentration of the mutated and control luciferase reporter plasmids was photometrically measured and was between 1.2 and 2.5µg/µl.

2.2.7.8 Transfections into Caco-2 cells

Caco-2 cells were grown to 80% confluence and plated in 24-well plates the day before transfection, with each well containing about 5x10⁴ Caco-2 cells. The medium was changed with 1ml fresh full culture medium before transfection.

Each transfection experiment was carried out in 4 or 8 fold repeats. A DNA mix was prepared for each well with 0.2µg luciferase reporter gene plasmid, 0.1µg pCMV-lacZ plasmid (for β-galactosidase expression, Promega), 0.9µg pBluescript SK⁺ plasmid (to

adjust the total DNA amount to 0.3µg, Stratagene) in a 25µl volume containing 15mM NaCl. 25µl of transfection reagent containing 2µM polyethyleneimine (PEI) in form of Exgen 500 (22kDa, Fermentas) in 120mM NaCl solution or PEI25 (25kDa, Alfa Aesar) were added to the DNA mix and incubated at room temperature for 1 hour.

The mixture was slowly added to the cells and the plates centrifuged for 5 minutes at 1200rpm to settle the reagent on the cell surface. The plates were incubated at 37°C and 5% CO₂. The medium was changed the first day after transfection and later when necessary.

2.2.7.9 Reporter gene assay analysis

After 2 and 9 days of transfection the Caco-2 cells were harvested at an undifferentiated and differentiated stage respectively and luciferase and β-galactosidase activity were measured using the Dual-Light System chemiluminescent reporter gene assay (ABI, Bronstein et al. 1997; Martin et al. 1996) following the 'luciferase and β-galactosidase detection protocol'.

The wells with the Caco-2 cell cultures were rinsed with 1ml room temperature PBS, which was completely removed by inverting the plate onto a paper towel and pipetting the remaining PBS from the side of the well. 130µl Lysis solution (containing 0.5mM DTT) was added to each well to cover the cells and after incubation for 5 minutes on a shaking platform the plates were placed on ice. 10µl of the cell extract was transferred to a luminometer vial. 25µl Buffer A was added to the bottom of the vial and it was placed in the luminometer immediately afterwards. 100µl Buffer B (containing luciferin and 1:100 Tropix Galacton-Plus substrate) was automatically added by the luminometer and the luciferase signal read for 5 seconds after a 2 second delay. The signal from the β-galactosidase reaction is negligible at that stage due to the lack of turnover time and an enhancer and a non optimal pH. The tubes were further incubated at room temperature for 30-60 minutes and 100µl Accelerator-II was added to raise the pH of the solution and provide a luminescence enhancer for β-galactosidase. After automatically adding of the Accelerator-II solution by the luminometer the luciferase signal was read again with a 2 second delay for 5 seconds.

Luciferase activity was normalised against β-galactosidase activity by calculating relative luciferase/β-galactosidase ratios for each well and significance between the transfection results tested with a student unpaired *t* test in Excel.

2.3 Statistical methods/Bioinformatic analyses

2.3.1 Deviation from Hardy-Weinberg equilibrium (HWE)

In a randomly mating population the expected genotype proportions from a determined allele frequency should follow the Hardy-Weinberg-theorem as they remain constant over generations in a stable population. The HWE is described by the formula: $p^2 + 2pq + q^2 = 1$, where p represents the major allele frequency and the minor allele frequency can be calculated by $q = 1 - p$.

Deviations from HWE might be observed due to factors like non-random mating, population stratification, presence of selection (though unlikely to be detected in small sample sets), deletions and duplications (CNV), or even technical issues with collecting and analysing the data in the lab. In this project, tests for deviation from HWE were mainly used to check data for errors caused by allelic dropout or sampling problems.

All genotype data from enhancer and haplotype markers were tested for deviation from HWE with the software Arlequin (version 3.5.1.3, Excoffier and Lischer 2010), which uses a kind of Fisher's exact test for each locus separately (Guo and Thompson 1992).

2.3.2 Haplotype inference

As all samples typed for this project are unrelated individuals, a statistical approach is needed to reconstruct haplotypes. The software PHASE (Stephens and Donnelly 2003; Stephens et al. 2001) applies a Bayesian statistical method to infer haplotypes from population genotype data. The programme uses a Markov chain-Monte Carlo algorithm, called Gibbs sampling, to calculate an expected pattern of haplotype distribution taking into account observed information of the dataset. Known haplotypes, occurring in homozygous individuals for example are fully informative and can be used for the calculation of unresolved haplotype pairs and those that are even then not resolvable are assumed more likely to include ones most similar to known haplotypes. The haplotypes for each individual are then reconstructed as most likely haplotypes estimated from the calculated posterior distributions by constructing a Markov chain.

Version 2.1.1 of the PHASE package (Stephens and Scheet 2005), which was used in this study, can additionally take into account order and genetic distances between the genotyped markers and the resulting estimated decay of linkage disequilibrium. The PHASE output was checked by eye for possible anomalies. Default settings were used for most of the analysis, only for extended haplotype analysis (chapter 5) the number of iterations was reduced to 50.

2.3.3 Haplotype networks

Haplotype information analysed by PHASE was used to construct a haplotype network with the software Network (version 4.6.1.1, <http://www.fluxus-engineering.com>). Allelic composition of each haplotype occurring at more than 4 chromosomes and haplotype frequencies were entered into the program and manually rooted to an ancestral haplotype (ancestral alleles inferred as described above, 2.2.4.6). The median-joining algorithm (Bandelt et al. 1999) was used to calculate the networks. It can deal with all types of data and constructs rooted networks with the shortest possible trees and reduces unnecessary cross links.

2.3.4 Linkage disequilibrium

Haplotype information was also used to estimate the level of linkage disequilibrium between alleles, which describes the non-random association between them (see also chapter 1). Pairwise linkage disequilibrium D between two alleles at different loci (A and B) of a haplotype is calculated with the formula $D = p_{AB} - (p_A \cdot p_B)$, in subtracting the observed haplotype frequency from this expected under linkage equilibrium when $p_{AB} = p_A \cdot p_B$ (whereas p is the frequency of one of the alleles at A and B). To be able to compare the values of two loci, the D parameter, which is highly dependent on allele frequencies, needs to be normalised. In dividing D by the theoretical maximum for the allele frequencies observed, D' can be calculated as proposed by Lewontin (1964). The values for D' lay between one (complete LD) and zero, values in between indicate a disruption of LD. Linkage disequilibrium was calculated using D' with PowerMarker (version 3.25, Liu and Muse 2005) and results were also checked in DnaSP (version 5.10.01, Librado and Rozas 2009). Both programs apply a chi-square test to assess the significance of associations between loci, whereas the Bonferroni corrected significance levels DnaSP calculates were used to mark significant values in the tables.

2.3.5 Linkage disequilibrium unit (LDU) maps

A high resolution LDU map for chromosome 2 was constructed with the help of Winston Lau using LDMap software (Lau et al. 2007) with a method that was initially developed by Maniatis et al. (2002) and extended by Morton et al. (2007). The method is based on the Malecot model which describes the isolation by distance of two SNPs. The probability (ρ) of an association between two SNPs is given by the modified Malecot equation (Morton et al. 2001):

$$\rho = (1 - L)Me^{-\sum \varepsilon_i d_i} + L,$$

where L is the association level at large distance (LD asymptote), M the association level at no distance (initial value before LD decays) and ε_i the decline of association which happens exponentially with physical distance d_i , measured in kb between the i th pair of SNPs. LDU distances between SNPs are given by $\varepsilon_i d_i$ that is equivalent to θt , the product of recombination θ and t the time in generations this recombination has accumulated after population bottleneck(s). In summary εd provides a more useful measure for LD as it is more accurately known than θt . LDU can also reflect effects of population bottleneck events that can cause differences in LD patterns between populations but it is also influenced by evolutionary forces such as selection and mutation (Tapper 2007). LD units for chromosome 2 were calculated by Winston Lau and values extracted for the region of interest. LDU were plotted as LDU/kb and LDU/Mb against distance (kb) in Excel.

2.3.6 Test for extended haplotype homozygosity (EHH)

Long range haplotype tests such as EHH tests use the principle that an allele under positive selection rises quickly in frequency and creates a region of extended homozygosity, as time would have been too short to break down LD of the region (details in chapter 1). The EHH test was applied in chapter 5 to compare haplotype data over a region of about 1.8 Mb, using Sweep software (version 1.1, Sabeti et al. 2002). EHH data for selected cores was exported in order to plot graphs of EHH decay using R, with a modified code from Pascale Gerbault.

2.3.7 Genetic distances between populations (F_{ST})

F_{ST} is a measure of genetic distances between subpopulations of a meta-population with divergence in time. It measures the deviation between the observed and expected heterozygote frequencies under the Hardy-Weinberg theorem. It takes into account that in a metapopulation the heterozygosity level is reduced with the divergence of subpopulations and in the subpopulations an excess of homozygotes appears, which is known as the Wahlund effect (Jobling et al. 2013). F_{ST} describes the differences between the average expected heterozygosity of sub-populations (H_S) and the expected total heterozygosity of the meta-population (H_T): $F_{ST} = (H_T - H_S) / H_T$. When high gene flow and little differentiation between the subpopulations are apparent, it is close to zero. It is close to one when the genetic diversity of the metapopulation is much higher than in any of the subpopulations, due to their high differentiation.

For comparison between two populations pairwise F_{ST} is calculated as $F_{ST}=V_p/p(1-p)$ with p and V being mean and variance of allele frequencies. Pairwise F_{ST} values were calculated with the Arlequin software (version 3.5.1.3, Excoffier and Lischer 2010).

2.3.8 Principal co-ordinates analysis (PCO)

The genetic distances between populations calculated as pairwise F_{ST} values were graphically represented in principal co-ordinates (PCO) plots (Gower 1966), in which multiple dimensions are reduced to be displayed on axes known as principal co-ordinates or eigenvectors.

These axes are extracted one after another to capture as much of the remaining variation as possible, with each axis being independent and orthogonal to the previous one. Plotted are the first two axes with the highest proportion of total variation that they account for. PCO plots were constructed using the R statistical environment (version 2.6.2., <http://www.r-project.org/>) using a code written by Christopher Plaster to convert the calculated pairwise F_{ST} values.

2.3.9 Diversity measures

Population diversity analyses were performed using DnaSP (version 5.10.01, Librado and Rozas 2009).

To prepare input files for these programs it was necessary to convert the haplotypes inferred by PHASE (and checked visually) to a full length haplotype sequences. Human reference sequences were downloaded as described in section 2.2.4.6. and manually edited and converted into FASTA format in BioEdit (version 7.0.9.0, Hall 1999) to only include the readable sequence length (559 bp) for the *LCT* enhancer region (chapter 3) and a combined sequence (1401 bp) of intron 4, enhancer and hapdef region (chapter 5). FASTA sequences for all haplotypes were created by assigning the nucleotides of a particular haplotype to their position on the FASTA reference sequences in using a Java code written by Maxim Scheremetjew. Individuals were assigned to their two haplotype sequences in using the most probable haplotype combination for each individual calculated by PHASE and connecting them to the haplotype FASTA sequences with Python using a code written by Mirna Kovacevic.

Haplotype diversity H (or gene diversity, heterozygosity) was measured using Nei's H (Nei 1987), which calculates the probability that two randomly taken sequences from a population are different from each other with the formula: $H = \frac{1}{n-1} (1 - \sum_{i=1}^K x_i^2)$.

Where n are the numbers of chromosomes (sample size), K are the number of different haplotypes and x_i the relative frequency of haplotype i . Values for H lie between zero and one, from no diversity to the most.

Another diversity measure calculated was the **nucleotide diversity π** (Nei, 1987). It is the probability that two randomly chosen sequences from a population differ at a certain site or simply the average number of nucleotide differences per site: $\pi = \frac{1}{n-1} \sum_{i,j}^K x_i x_j d_{ij}$.

Where n is the numbers of chromosomes (sample size), K is the number of different haplotypes, x_i and x_j are the respective frequencies of the i th and j th sequences, d_{ij} is the number of nucleotide differences per nucleotide site between sequences i and j .

Both formulas calculate values that are corrected for sample size.

2.3.10 Tests of neutrality

Several statistics were applied to compare the observed diversity patterns with these expected under neutral evolution (see also chapter 1). Neutrality tests were conducted with DnaSP using phased haplotype data.

Tajima's D (Tajima 1989) calculates the genetic diversity within a population in comparing each pair of sequences in taking into account the number of segregating sites (S) and the average number of nucleotide diversity (π), and compares that to the expected level of diversity under neutrality. Significant negative Tajima's D values indicate positive selection or population growth and positive values could indicate population subdivision or balancing selection (Jobling et al. 2013). Tajimas' D should be zero under neutrality. P -values for the significance of the Tajima's D values deviating from zero are also calculated by DnaSP.

Fu and Li's D^* and F^* statistics (Fu and Li 1993) were also applied, which test for an excess of rare variants in the populations. The D^* test measures the proportion of singletons compared to the total number of mutations in a population. The F^* statistics additionally compares the number of singletons to the average number of nucleotide differences between sequence pairs in a population. DnaSP indicates the statistical significance of both values in taking the critical values obtained by a two tailed test (Fu and Li 1993).

2.3.11 GenoPheno

To be able to compare the predicted phenotype data for the different populations in chapter 3, estimated from frequencies of lactase persistence associated alleles, with published lactase persistence phenotype data of matching populations, the GenoPheno test (Mulcare et al. 2004) was applied. GenoPheno, version 1.00, is an R script written by Dr. Mike Weale and it is accessible via the TCGA website (<http://www.ucl.ac.uk/tcga/software/>). This method takes sampling errors for genotype and phenotype data (summarised in Mulcare et al. 2004) into account and statistically tests the discrepancy of predicted and observed phenotype data.

To briefly describe the method, the predicted frequency of lactose digesters is calculated from the combined frequencies of the associated alleles ($p^2+2p(1-p)$) and corrected for different kinds of phenotyping error. Then a value for the number of lactose digesters is simulated from a Binomial distribution taking into account the numbers of predicted digesters and observed digesters. These steps are repeated 100,000 times to get a Monte Carlo sampling distribution of the numbers of observed digesters under the null hypothesis that genotype data and phenotyping error alone account for the predicted lactose digester frequencies. From this simulated distribution a two-tailed p -value is obtained.

To create the Old-World genotype-phenotype correlation map the approach of Itan et al. (2010) was followed. The program, written in the Python programming language, uses the statistical GenoPheno method described above and combines that with surface interpolation to cover areas with missing data points. Information about genotype data collection points by the group or taken from literature were converted into geographic co-ordinates in using Google Earth (<http://www.google.com/earth/index.html>). The genotype-phenotype correlation maps were plotted with the help of Pascale Gerbault and Yuval Itan using the modified Python code written by Yuval Itan (Itan et al. 2010).

2.3.12 Fisher's exact test

The significance of associations between loci of intron 2 of *LCT* and the lactase persistence phenotype (chapter 6) was tested with Fishers exact test. A 2x2 contingency table was generated with the observed allele counts at a locus and the existing phenotype data. The test calculates the differences between the observed and expected values under the null hypothesis that values of rows and columns are not associated. The Graphpad online

software (<http://graphpad.com/quickcalcs/contingency1.cfm>) was used to calculate two tailed *p*-values for the Fishers exact tests.

2.3.13 Prediction of transcription factor binding sites

The bioinformatic search for potential transcription factor binding sites was done with several different software programs that use different mathematical algorithms to compare the input sequence to partly different libraries of known regulatory motifs. All results were checked for relevance in filtering for tissue and cell type specificity where possible.

The **MATCH** tool (Kel et al. 2003) uses the TRANSFAC database (TRANSFAC Professional version 12.1, BIOBASE Biological Databases) (Matys et al. 2006). With this software it is possible to compare DNA sequences with a library of mononucleotide weight matrices, matching possible transcription factors. The search can be additionally specified by species or tissue profiles. For the analyses done for this project, a vertebrate matrix was chosen with a cut-off value that reduces false positive and false negative results. The quality of a match is defined by the core similarity score (core match) and the matrix similarity score (matrix match). The threshold for the matrix score/match was set to 80% to avoid false negative results.

The freely online available tool **TFSEARCH** (version 1.3, <http://www.cbrc.jp/research/db/TFSEARCH.html>) also uses the TRANSFAC database, but with a slightly different algorithm to match the database entries. No information about the exact method was available. The program was used for comparison purposes.

MatInspector (version 8.0.5) accesses its own database (Cartharius et al. 2005). The software uses a similar scoring scheme as MATCH to compare a given sequence with the position weight matrices of the transcription factor binding sites of the database but additionally works with family matrices. The program calculates a matrix similarity score of a certain sequence to the matching matrices of the library consisting of information about the position weight matrix, a conservation profile and a core region for a transcription factor. The matrices of the program are also stored as family matrices to minimise redundant matches.

2.3.14 Further statistical tests or methods

Spearman Rank correlation tests were performed in Excel to analyse correlations between allele frequencies and geographic locations of populations. Therefore the distances between the assigned geographic coordinates for the population and 10°W longitude and 80°N latitude were calculated and converted in km using a converter online program (<http://www.movable-type.co.uk/scripts/latlong.html>).

Mann-Whitney U tests were performed online (<http://elegans.som.vcu.edu/~leon/stats/utest.cgi>). Mean lengths of haplotypes carrying derived and ancestral alleles were calculated in bp and tested with a two tailed test.

The geographic map showing the lactase persistence frequencies in chapter 1 was plotted with data from Itan et al. (Itan et al. 2010) by Mark Thomas using a Python scripting. The other geographic map showing enhancer alleles distribution in chapter 3 was generated in R (version 3.0.1) using a modified code written by Pascale Gerbault.

2.4 Web resources

Affymetrix: <http://www.affymetrix.com/estore/>
Arlequin: <http://cmpg.unibe.ch/software/arlequin35/>
BioEdit: <http://www.mbio.ncsu.edu/bioedit/bioedit.html>
ChromasPro: <http://technelysium.com.au/>
Centre for Comparative genomics: <http://www.ucl.ac.uk/gee/research/CCG>
CEPH: <http://www.cephb.fr/en/hgdp/diversity.php>
DnaSP: <http://www.ub.edu/dnasp/>
DoubleDigest: <http://www.thermoscientificbio.com/webtools/doubledigest/>
Ensembl: <http://www.ensembl.org/index.html>
Ethnographic Atlas: <http://eclectic.ss.uci.edu/~drwhite/worldcul/atlas.htm>
Ethnologue: <http://www.ethnologue.com/>
FastPCR: <http://primerdigital.com/fastpcr.html>
GLAD database: <http://www.ucl.ac.uk/mace-lab/resources/glad>
Google Earth: <http://www.google.com/earth/index.html>
Graphpad: <http://www.graphpad.com/>
HapMap: <http://hapmap.ncbi.nlm.nih.gov/>
MatInspector: <http://www.genomatix.de/solutions/genomatix-software-suite.html>
NCBI: <http://www.ncbi.nlm.nih.gov/>
Phase: <http://stephenslab.uchicago.edu/software.html>
Powermarker: <http://statgen.ncsu.edu/powermarker/>
R: <http://www.r-project.org/>
Sweep: <http://www.broadinstitute.org/mpg/sweep/>
SNPviewer: <http://www.lgcgenomics.com/software>
TCGA: <http://www.ucl.ac.uk/tcga/software/>
TFSEARCH: <http://www.cbrc.jp/research/db/TFSEARCH.html>
The intestinal transcription factor target database:
<http://gastro.sund.ku.dk/chipchip/>
TRANSFAC: <http://www.biobase-international.com/product/transcription-factor-binding-sites>
UCSC: <http://genome.ucsc.edu/>
UniProt: <http://www.uniprot.org/>
Vector NTI: <http://www.lifetechnologies.com/uk/en/home/life-science/cloning/vector-nti-software.html>

2.5 Laboratory chemicals and equipment

2.5.1 Labmade solutions

All pH values are at 25°C.

2/3 Microclean (HM-MC): 26.7% Polyethylene glycol (PEG 8000), 0.7M NaCl, 1.3mM Tris-HCl (pH 7.5), 0.13mM EDTA (pH 8.0), 2.3mM MgCl₂

TBE buffer (1x): 89mM Tris-HCl (pH8.0), 89mM boric acid, 2mM EDTA (pH 8.2-8.4)

TE buffer (1x): 10mM Tris-HCl (pH 8.0), 1mM EDTA (pH 8.0)

Agarose gel loading buffer: 15% Ficoll (PM400) in H₂O, traces (about 0.25%) of Xylene cyanol FF (Sigma) and Bromophenol blue (Bio-Rad)

Slagboom buffer: 10mM EDTA (pH8.0), 100mM NaCl, 10mM Tris-HCl (pH8.0), 0.5% SDS and 0.2mg/ml Proteinase K (Sigma)

Stretch marker: Agarose gel loading buffer containing about 50ng 200, 400 and 800 bp PCR products

Dialysis buffer: 20mM HEPES (pH 7.9), 20% glycerol, 1.5mM MgCl₂, 100mM KCl, 0.2mM EDTA, 0.5mM dithiothreitol (DTT)

EMSA loading buffer: 0.2% Bromophenol blue, 10% glycerol, 0.5xTBE buffer

Gel shift buffer: 25mM Tris-HCl (pH 7.8), 5mM MgCl₂, 6mM KCl, 0.5mM EDTA, 1mM DTT, 1µl/ml protease inhibitor cocktail (Sigma), 5%Ficoll (PM 400), 2.5% glycerol

High-salt buffer: 20mM HEPES (pH 7.9), 25% glycerol, 1.5mM MgCl₂, 1.2M KCl, 0.2mM EDTA, 0.5mM DTT, 1µl/ml protease inhibitor cocktail

Hypotonic buffer: 10mM HEPES (pH 7.9), 1.5mM MgCl₂, 10mM KCl, 0.2mM PMSF, 0.5mM DTT

Low-salt buffer: 20mM HEPES (pH 7.9), 25% glycerol, 1.5mM MgCl₂, 0.2M KCl, 0.2mM EDTA, 0.5mM DTT, 1µl/ml protease inhibitor cocktail

2.5.2 Commercial solutions

All pH values are at 25°C

LB Agar: 9.14g/L tryptone, 4.57g/L yeast extract, 4.57g/L NaCl, 13.72g/L agar, 1.6g/L inert tableting aids

LB medium: 10g/L enzymatic digest of casein, 5g/L yeast extract (low sodium), 5g/L sodium chloride, 2g/L inert agents, 22g/L total solids

Advantage-GC cDNA PCR Kit

Advantage-GC cDNA Polymerase Mix, recombinant: KlenTaq-1 DNA polymerase and TaqStart Antibody (1.1µg/µl) in 1x buffer: 1.0%Glycerol, 0.8mM Tris-HCl (pH 7.5), 1.0mM

KCl, 0.5mM (NH₄)₂SO₄, 2.0 μM EDTA, 0.1mM β -mercaptoethanol, 0.005%Thesit, Deep Vent_R as minor component

1x GC cDNA PCR reaction buffer: 40mM Tricine-KOH (pH 9.2 at 25°C), 15mM KOAc, 3.5mM Mg(OAc), 2, 5% Dimethyl Sulfoxide (DMSO), 3.75 μg/ml μg/ml Bovine serum albumin
dNTP mix (1x): (10mM each of dATP, dCTP, dGTP, and dTTP; 1x concentration: 0.2mM each)

TOPO-TA Cloning kit

pCR 2.1-TOPO, 10 ng/μl plasmid DNA in: 50% glycerol, 50mM Tris-HCl (pH 7.4), 1mM EDTA, 1mM DTT, 0.1% Triton X-100, 100μg/ml BSA, phenol red

Salt Solution: 1.2M NaCl, 0.06M MgCl₂

S.O.C. medium: 2% Tryptone, 0.5% Yeast Extract, 10mM NaCl, 2.5mM KCl, 10mM MgCl₂, 10mM MgSO₄, 20mM glucose

Restriction enzymes and buffers

BamHI in 1x buffer: 10mM Tris-HCl (pH 7.4 at 25°C), 200mM NaCl, 1mM DTT, 0.1mM EDTA, 0.15% Triton X-100, 0.2mg/mL BSA, and 50% (v/v) glycerol

Buffer BamHI (1x): 10mM Tris-HCl (pH 8.0 at 37°C), 5mM MgCl₂, 100mM KCl, 0.02% Triton X-100, and 0.1mg/mL BSA

Pfu DNA polymerase, recombinant, in 1x buffer: 20mM Tris-HCl (pH 8.2), 1mM DTT, 0.1mM EDTA, 100mM KCl, 0.1% (v/v) Nonidet P40, 0.1% (v/v) Tween 20, and 50% (v/v) glycerol

Pfu Buffer with MgSO₄ (10x): 200mM Tris-HCl (pH 8.8), 100mM (NH₄)₂SO₄, 100mM KCl, 1mg/mL BSA, 1% (v/v) Triton X-100, 20mM MgSO₄.

T4 DNA ligase in 1x buffer: 20mM Tris-HCl (pH 7.5), 50mM KCl, 1mM DTT, 0.1mM EDTA and 50% (v/v) glycerol

T4 DNA Ligase Buffer 10x: 400mM Tris-HCl, 100mM MgCl₂, 100mM DTT, 5mM ATP (pH 7.8)

T4 Polynucleotide Kinase in 1x buffer: 20mM Tris-HCl (pH 7.5), 25mM KCl, 0.1mM EDTA, 2mM DTT and 50%(v/v) glycerol

Kinase reaction buffer A (10x): 500mM Tris-HCl (pH 7.6), 100mM MgCl₂, 50mM DTT, 1mM spermidine

Taq DNA polymerase in 1x buffer: 100mM KCl, 20mM Tris-HCl, pH 8.0, 0.1mM EDTA, 1mM DTT, 0.5% Tween 20, 0.5% Nonidet P40, 50% (v/v) glycerol

Reaction Buffer IV (10x): 750mM Tris-HCl (pH 8.8), 200mM (NH₄)₂SO₄, 0.1% (v/v) Tween 20

SacI, Sall and XhoI in 1x buffer: 10mM Tris-HCl (pH 7.4), 100mM KCl, 1mM DTT, 1mM EDTA, 0.2mg/mL BSA, 50% (v/v) glycerol

Tango Buffer (1x): 33mM Tris-acetate (pH 7.9 at 37°C), 10mM Mg-acetate, 66mM K-acetate, 0.1mg/mL BSA

2.5.3 Equipment

Centrifuges, general

ALC Centrifuge PK120

Biofuge Pico, Heraeus

Capsule Tomy HF-120

Centrifuge 5430, Eppendorf

MSE-Europa 24M, SANJO

MSE MISTRAL 3000g, SANJO

Sigma 1-14

EMSA

Ole Dich Inmicrocentrifuge 157 MP

SE 250Mighty small II Electrophoresis, Hoefer Scientific Instr.

Multitemp II, Thermostatic circulator, Pharmacia LKB

SlabGEL Dryer SGD4050, Savant

GelPump GP100, Savant

Storage Phosphor Screen 20x25, Molecular dynamics, Amersham Biosciences

Image Eraser, Molecular dynamics, Amersham Biosciences

Storm Phosphorimager 820

Series 900 radiation mini-monitor G-M (Coderum) tube, Morgan Mini Instruments Ltd

Microbiological work

Centrifuges: Jouan CR 312, Sigma 3-18K, Sorovall SS-34

Heraeus UB 20 incubator

Lauda E100 Ecoline waterbath, VWR

Teche Hybridiser HB-ID

Berthold Lumat LB9501 Tube Luminometer

Other equipment

ABI GeneAmp PCR System 9700

ABI Veriti 96 well Thermal Cycler

GeneQuant II photometer, Pharma Biotech, Printer EPSON P-40

BioPhotometer 6131, Eppendorf

NanoDrop 1000 Spectrophotometer and -software, version 3.8.1

Luckham 4RT Rocking platform

G:BOX EF2, Syngene, Software GeneSnap and GeneTools, Syngene

UVP UV Transilluminator

2.5.4 Suppliers

AH diagnostics as (Stratagene): Runetoften 18, 8210 Aarhus V, DK

Autogen Bioclear UK Ltd: Holly Ditch Farm, Mile Elm, Calne SN11 0PY, UK

BioGenex: 49026 Milmont Drive, Fremont, California 94538, USA.

BioNordika Denmark A/S: Marielundvej 48, 2730 Herlev, DK

Bio-Rad Laboratories: Symbion Science Park, Fruebjergvej 3, 2100 København ø, DK

CareFusion Health UK 232 Ltd (Micro Medical Ltd): The Crescent, Jays Close, Basingstoke RG22 4BS, UK

Eurofins MWG Operon: Anzinger Str. 7a, 85560 Ebersberg, Germany

Fisher Scientific (Fermentas, Lonza Bioscience, Macherey-Nagel): Postboks 60, Industrivej 3, 3550 Slangerup, DK

Fisher Scientific UK Ltd: Bishop Meadow Road, Loughborough LE11 5RG, UK

GE Healthcare Europe GmbH (Amersham Biosciences): Park Allé 295, 2605 Brøndby DK

Johnson Matthey A/S (Alfa Aesar): Frederikssundsvej 274 D, 2700 Brønshøj, DK

LGC Genomics Ltd (KBioscience): Units 1 & 2, Trident Industrial Estate, Pindar Road, Hoddesdon EN11 0WZ, UK

Life Technologies Ltd (Applied Biosystems): 3 Fountain Drive, Inchinnan Business Park, Paisley PA4 9RF, UK

Life Technologies Europe BV (Invitrogen): filial Danmark, PO Box 37, 2850 Naerum, DK

Medicell International Ltd: 239 Liverpool Road, London N1 1LX, UK

Perkin Elmer: Tonsbakken 16-18, Skovlunde 2740, DK

QIAGEN (Gentra): Skelton House, Lloyd Street North, Manchester M15 6SH, UK

Santa Cruz Biotechnology Inc: 2145 Delaware Avenue, Santa Cruz, California 95060, USA

Sigma-Aldrich Company Ltd: The Old Brickyard, New Road, Gillingham SP8 4XT, UK

Thermo Fisher Scientific (previous ABgene): Abgene House Blenheim Road, Epsom KT19 9AP, UK

VWR: Bie & Berntsen A/S: Transformervej 8, 2730 Herlev, DK

VWR International Ltd: Hunter Boulevard, Magna Park, Lutterworth LE17 4XN, U

3 Geographic distribution of LCT enhancer variation in Eurasian populations

3.1 Introduction

As described in chapter 1, the continued expression of lactase into adulthood is enabled by a mechanism *cis*-acting to *LCT* (Wang et al. 1995). A relevant regulatory region was found in intron 13 of the upstream gene *MCM6* that showed influence on *LCT* promoter activity with a transcriptional enhancing effect (Olds and Sibley 2003; Troelsen et al. 2003).

Within this sequence region, the allele *-13910*T* was shown to nearly completely associate with lactase persistence, in Northern Europe (Enattah et al. 2002; Enattah et al. 2007; Poulter et al. 2003). *-13910C>T* genotypes were also associated with a trimodal lactase expression pattern in a study on Finns, as one would expect from a *cis*-acting element (Kuokkanen et al. 2003). This general pattern was also seen by Poulter et al. (2003), although the pattern was not so clear-cut. This allele is also present at considerable frequencies in groups of European descent in America or African groups with a known history of admixture with Europeans or Asians (for example Friedrich et al. 2012; Myles et al. 2005).

The absence of *-13910*T* in sub-Saharan African and Middle Eastern populations where the lactase persistence phenotype is common (Ingram et al. 2007; Mulcare et al. 2004) led to further investigations involving re-sequencing the *LCT* enhancer region, which revealed more variation. Some of these alleles were too rare to assess for association but three were shown to associate with lactase persistence, for example *-13915*G*, which is frequent in the Middle East and East Africa (Enattah et al. 2008; Imtiaz et al. 2007; Ingram et al. 2007; Ingram et al. 2009b; Tishkoff et al. 2007). This allele is associated with lactase persistence in populations from Saudi Arabia (Imtiaz et al. 2007), Kenya (Tishkoff et al. 2007), in the Jaali of Sudan (Ingram et al. 2007) and Somali of Ethiopia (Ingram et al. 2009b).

In the same Somali, another allele, *-13907*G*, is also associated with lactase persistence (Ingram et al. 2009b) and was shown to be common in several East African populations (Tishkoff et al. 2007).

About 100 bp upstream and occurring at high frequencies in several populations from Kenya and Tanzania another allele, *-14010*C* showed association with lactase persistence too (Tishkoff et al. 2007). It is also relatively common in the South Africa Xhosa (Torniainen et al. 2009). A further allele, *-14009*G*, was associated in the Somali but did not reach statistical significance (Ingram et al. 2009b).

3.2 Chapter aims

Analysis of the published data on the frequencies of the four confirmed associated alleles did not fully explain the distribution of the lactase persistence phenotype.

The approach by Itan et al. (2010), mentioned in chapter 1 (see also Figure 1.7), illustrates this: The geographic mapping of the difference in phenotype distribution predicted from genotype information of all four alleles with published observed lactase persistence phenotype data showed that there are many geographic regions where the lactase persistence phenotype is underrepresented by the genetic data. Whether caused by yet undiscovered variants, data errors or simply due to a lack of sampling points, as the map shows interpolated values, could not be clarified. Of these regions, Eastern Europe and parts of West, Central and South Asia, are of special interest for this thesis.

Although much was known about the worldwide occurrence of *-13910*T* at the onset of this thesis, and even though reasoned to be causal for lactase persistence in Europe, it could not completely explain the phenotype distribution in parts of South and East Europe (Anagnostou et al. 2009; Itan et al. 2010). These regions were not well covered with information about enhancer SNP data and furthermore, if reported at all, the *-13910 C>T* SNP had been mainly studied via RFLP or other genotyping technologies. Only a few studies provided data from fully sequenced enhancer regions before the beginning of this thesis (Enattah et al. 2008; Imtiaz et al. 2007; Ingram et al. 2007; Ingram et al. 2009b).

Two further examples suggested that the information about lactase persistence alleles in Europe was not complete. One variant, *-13914 G>A* had been reported in two individuals, from Germany and Austria (Tag et al. 2008; Tag et al. 2007) and four family members from Northern Russia with partly Polish ancestry (Khabarova et al. 2010). Another European individual showed a chromosome with high lactase expression (Poulter et al. 2003) and carrying a new enhancer variant, *-14028*C*, but none of the others (Ingram 2008). The occurrence of this variant in particular, in other individuals, would be of great interest as it showed direct evidence of function.

The three other reported lactase persistence associated alleles -13907*G, -13915*G and -14010*C had mainly been described in African and Middle Eastern populations (Enattah et al. 2008; Imtiaz et al. 2007; Ingram et al. 2007; Ingram et al. 2009b; Tishkoff et al. 2007) and not much was known about their distribution elsewhere. Also with respect to other recently discovered variants (for example Ingram et al. 2009b; Tag et al. 2007) further investigations on Eurasian and Middle Eastern populations seemed to be necessary to complete the picture of the distribution of known *LCT* enhancer alleles and to identify new variants. It was thus of interest to obtain complete enhancer sequence data in these areas. Any novel variants could be subjected to genotype-phenotype association study if at sufficient frequency or could be tested *in vitro* for function.

This chapter aims to describe the variation of enhancer alleles and its distribution in Europe, the Middle East and other parts of Asia. In summary, the main questions to answer are:

- What is the distribution of the European -13910*T allele and what is the edge of this distribution in southern and southeastern parts of Europe and in the Middle East?
- Can other lactase persistence associated or derived alleles be found in the enhancer region of *LCT* in the available samples and what is their distribution?
- Can the distribution of lactase persistence associated *LCT* enhancer alleles now explain the phenotypic distribution of reported groups in the same geographic areas?

3.3 Sequencing strategy

To examine the variation in the *LCT* enhancer present in populations from all over Europe, the Middle East and other parts of Asia, DNA samples were sequenced with the classic Sanger method and analysed (see section 2.2.4). Several samples previously genotyped by our group for -13910 C>T (Mulcare 2006) or haplotype markers (Hollox et al. 2001; Poulter et al. 2003) were also re-analysed via sequencing to ascertain the whole enhancer. Of these, samples from India and Southeast Asia were sequenced as part of an undergraduate project (Ghosh 2010).

A combination of four primers successfully applied in previous studies (Ingram et al. 2007; Ingram et al. 2009b) was first used to amplify the 940 bp region in intron 13 of *MCM6* including the *LCT* enhancer (Chr2:136608194-136609133, GRCh37/hg19), and sequences were readable from bp position -14331 until -13489 from the start of transcription of *LCT*.

Depending on the quality of the DNA it was sometimes necessary to amplify two shorter overlapping fragments to get sufficient amounts of products. Details about primer sequences can be found in Table 2.3 of chapter 2.

During the later part of this project the focus was more on the smaller region 3' in *MCM6* (Chr2:136608194-136608899) which was typed for all samples. Of this 706 bp long PCR product, it was possible to analyse the enhancer sequences of most of the samples for a 559 bp region from position -14040 to -13482 from the start of transcription of *LCT*.

3.4 Grouping strategy

The definition of groups for population genetic analyses is always to some extent artificial as it depends on the information available. Nevertheless it is essential to aim as far as possible for uniformity of groups in the analyses. For this project, the individual samples were first grouped according to the geographic region, namely Northwest/Central Europe, South Europe, East/Southeast Europe, the Middle East, West Asia, Central/South Asia and Central/East/Southeast Asia, and the country they were collected. Samples that could not be allocated to one country of that geographic region were classified as 'other'. These samples were not included in the geographic maps (see below). A further differentiation into distinct ethnic groups with a minimum sample size of 10 followed, using self declared ethnic background if such information was available or in sub-groupings within countries with further information about the sample localization. Otherwise they were grouped by country only.

3.5 Variation of the *LCT* enhancer

The *LCT* enhancer region in intron 13 of *MCM6* was successfully sequenced for 2056 individuals from 52 populations within this project. A total of 25 SNPs was identified, of which 10 occurred more than once. Table 3.1 shows these enhancer variants and the allele frequencies of non-singleton SNPs for each population. 9 new variants were identified (-14145 T>G, -14062 G>A, -14010 G>A, -13964 C>A, -13926 A>C, -13950 A>G, -13771 A>G, -13744 C>G, -13693 G>A) which are mainly singletons. They were confirmed by sequencing of both strands of the products of at least two independent PCRs and the ancestral state of these SNPs was determined by sequence comparison with other primate species, which was the same as the common allele in humans (see also chapter 2, section 2.2.4.6). Sequencing chromatograms of some of the new variants are shown in Figure 3.1.

All SNPs were checked for deviation from HWE using the Arlequin software (see also chapter 2, section 2.3.1). A few significant deviations were observed but after Bonferroni

correction for multiple testing (51 tests) only one, -13910C>T in the Yakuts ($p=0.0006$) remained significant. An underrepresentation of heterozygotes at this position was observed which could be the result of population substructure within the group sampled. After reassessment of the sampling locations (see also chapter 2, section 2.1.1.5) the group was divided into Northern Yakuts and Yakuts which left both groups in HWE.

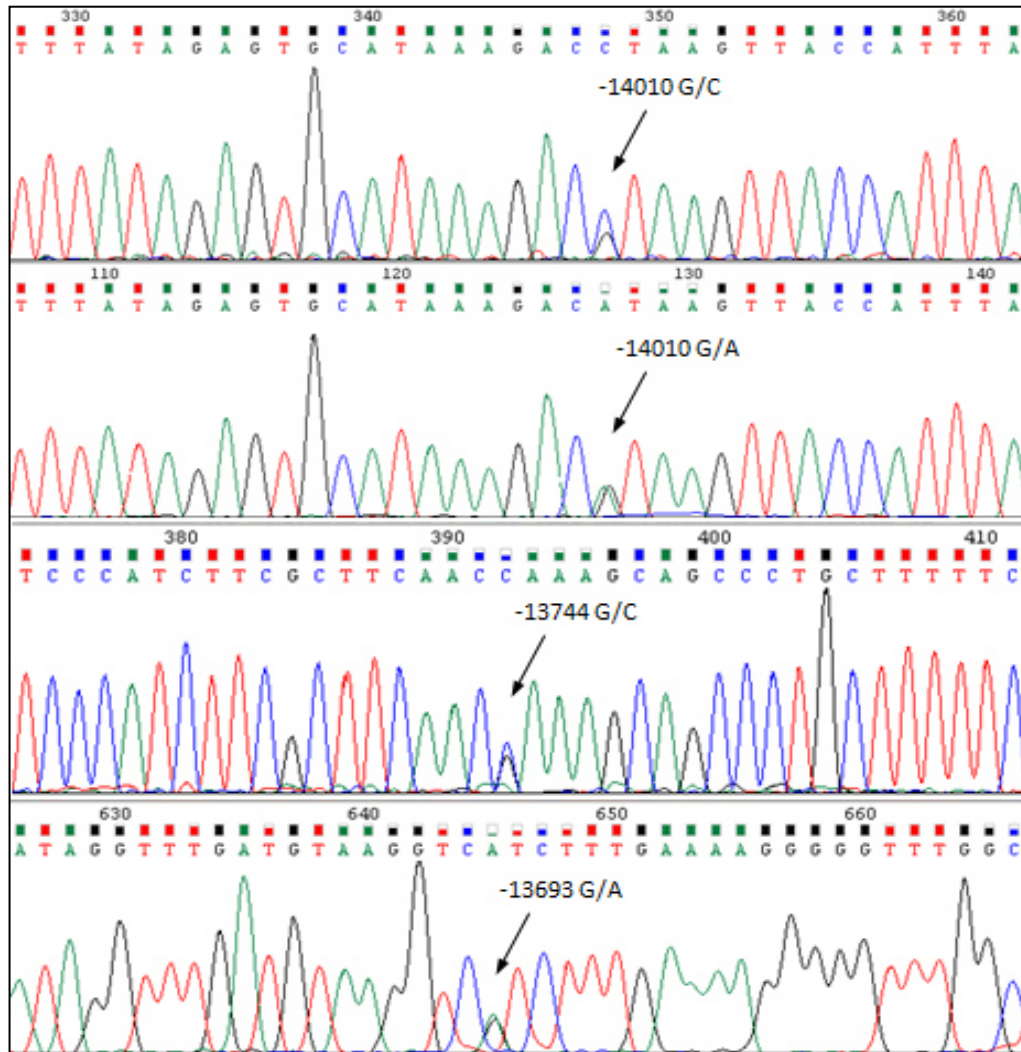


Figure 3.1: Sequence chromatograms showing examples of individuals heterozygous at various positions of the *LCT* enhancer. Note that the second chromatogram shows a third allele at the 14010 position which is known to be a G>C SNP (first chromatogram).

Table 3.1: LCT enhancer variation in the samples sequenced from 52 European and Asian population groups. Allele frequencies for all variants occurring more than once and the presence of singletons are indicated. N: number of chromosomes, data marked by * was not included in the geographic map (Figure 3.2).

Region	Country	Population	N	-14011 C>T	-13915 T>G	-13910 C>T	-13907 C>G	-13906 T>A	-13779 G>C	-13744 C>G	-13730 T>G	-13603 C>T	-13495 C>T	Singletons
Northwest/ Central Europe	Germany	Germans	60	-	-	0.550	-	-	-	-	-	-	0.617	
		Sorbs	64	-	-	0.500	-	-	-	-	-	-	0.617	
	Ireland	Irish	68	-	-	0.956	-	-	-	-	-	-	0.969	
	Netherlands	Frisians	58	-	-	0.810	-	-	-	-	-	-	0.845	
	Norway	Norwegians	88	-	-	0.841	-	-	-	-	-	-	0.875	
	Slovakia	Roma	64	-	-	0.063	-	-	-	-	-	-	0.400	
	Sweden	Sami	60	0.033	-	0.293	-	-	-	-	-	-	0.750	
		Swedes	74	-	-	0.778	-	-	-	-	-	-	0.906	
	UK	Ashkenazi-Jews	38	-	-	0.105	-	-	-	-	-	-	0.158	-13914 G>A
		English	102	-	-	0.745	-	-	-	-	-	-	0.784	-14062 G>A, - 14028 T>C
	Other*	other C/NW Europeans	68	-	-	0.691	-	-	-	-	-	-	0.727	
South Europe	Greece	Greeks	120	0.008	-	0.150	-	-	-	-	0.008	-	0.306	
	Italy	Tyroleans Bozen	80	0.013	-	0.150	-	-	-	-	-	-	0.368	
		Tyroleans Gadertal	76	-	-	0.421	-	-	-	-	-	-	0.514	
		Tyroleans Vinschgau	102	-	-	0.363	-	-	-	-	-	-	0.521	
	Portugal	Portuguese	96	-	-	0.438	-	-	-	-	-	-	0.486	
	Spain	Catalans	58	-	-	0.259	-	-	-	-	-	-	0.364	
		Spanish	62	-	-	0.403	-	-	-	-	-	-	0.500	
East/ Southeast Europe	Belarus	Belarusians	100	0.010	-	0.230	-	-	-	-	-	-	0.477	
	Macedonia	Macedonians	100	-	-	0.240	-	-	-	-	-	-	0.398	
	Romania	Romanians	118	-	-	0.169	-	-	-	-	-	-	0.278	-13753 C>T
	Russia	Erzya	42	-	-	0.143	-	-	-	-	-	-	0.333	
		Moksha	32	0.031	-	0.406	-	-	-	-	-	-	0.531	
		Russians Perm	46	-	-	0.239	-	-	-	-	-	-	0.395	
	Ukraine	Ukrainians	74	0.014	-	0.257	-	-	-	-	-	-	0.379	
	Other*	Other E/SE Europeans	38	-	-	0.289	-	-	-	-	-	-	0.444	
Middle East	Cyprus	Greek Cypriots	120	-	-	0.008	-	-	-	-	-	-	0.269	

West Asia	Iran	Iranians	154	0.006	0.006	0.032	-	-	-	-	-	-	0.234	-14009 T>G, -13693 G>A -14145 T>G -14010 G>C, -13806 A>G
	Kuwait	Kuwaiti	66	-	0.250	0.030	-	-	-	-	0.015	0.015	0.141	
	Syria	Syrians	140	-	0.029	0.021	-	-	0.007	-	-	-	0.231	
	Turkey	Anatolian-Turks	116	-	-	0.078	-	-	-	-	-	-	0.225	
	Yemen	Yemeni Hadramaut	166	-	0.241	0.018	0.012	-	0.030	0.006	0.006	-	0.210	
		Yemeni Sena	68	-	0.294	0.147	-	-	-	0.059	0.029	0.059	0.294	
	Other*	Other Middle Easterns	62	-	0.048	0.065	0.016	-	-	-	-	-	0.345	
	Armenia	Armenians	102	-	-	0.176	-	-	-	-	-	-	0.333	
	Azerbaijan	Azeri	80	-	-	0.013	-	-	-	-	-	-	0.171	
	Georgia	Georgians	108	-	-	0.075	-	-	-	-	-	-	0.293	
Central/ South Asia	Afghanistan	Pashtuns/Afghans	32	-	-	0.063	-	-	-	-	-	-	0.375	-13964 C>A
		Tadjiks	28	-	-	0.250	-	-	-	-	-	-	0.464	
		Uzbeks	54	-	-	0.241	-	-	-	-	-	-	0.463	
	India	North Indians	120	-	-	0.200	-	-	-	-	-	-	0.410	
		South Indians	102	-	-	0.118	-	-	0.029	-	-	-	0.450	
	Nepal	Nepalese	38	-	-	0.342	-	0.026	-	-	-	-	0.526	
		Tharu	80	-	-	0.175	-	-	-	-	-	-	0.321	
	Uzbekistan	Uzbeks	76	-	-	0.039	-	-	-	-	-	-	0.269	
	Other*	Other C/South Asians	32	-	-	0.188	-	-	-	-	-	-	0.500	
Central/East/ Southeast Asia	Mongolia	Khalka	114	0.009	-	0.026	-	-	-	-	-	-	0.413	
		Mongols	52	-	-	0.038	-	-	-	-	-	-	0.417	-13771 A>G -14010 G>A, -13926 A>C
	Russia	Northern Yakuts	22	-	-	0.136	-	-	-	-	-	-	0.500	
		Yakuts	110	-	-	0.055	-	-	-	-	-	-	0.443	
	Singapore	Han-Chinese	98	-	-	0.041	-	0.010	-	-	-	-	0.382	
		Japanese	84	-	-	-	-	-	-	-	-	-	0.333	

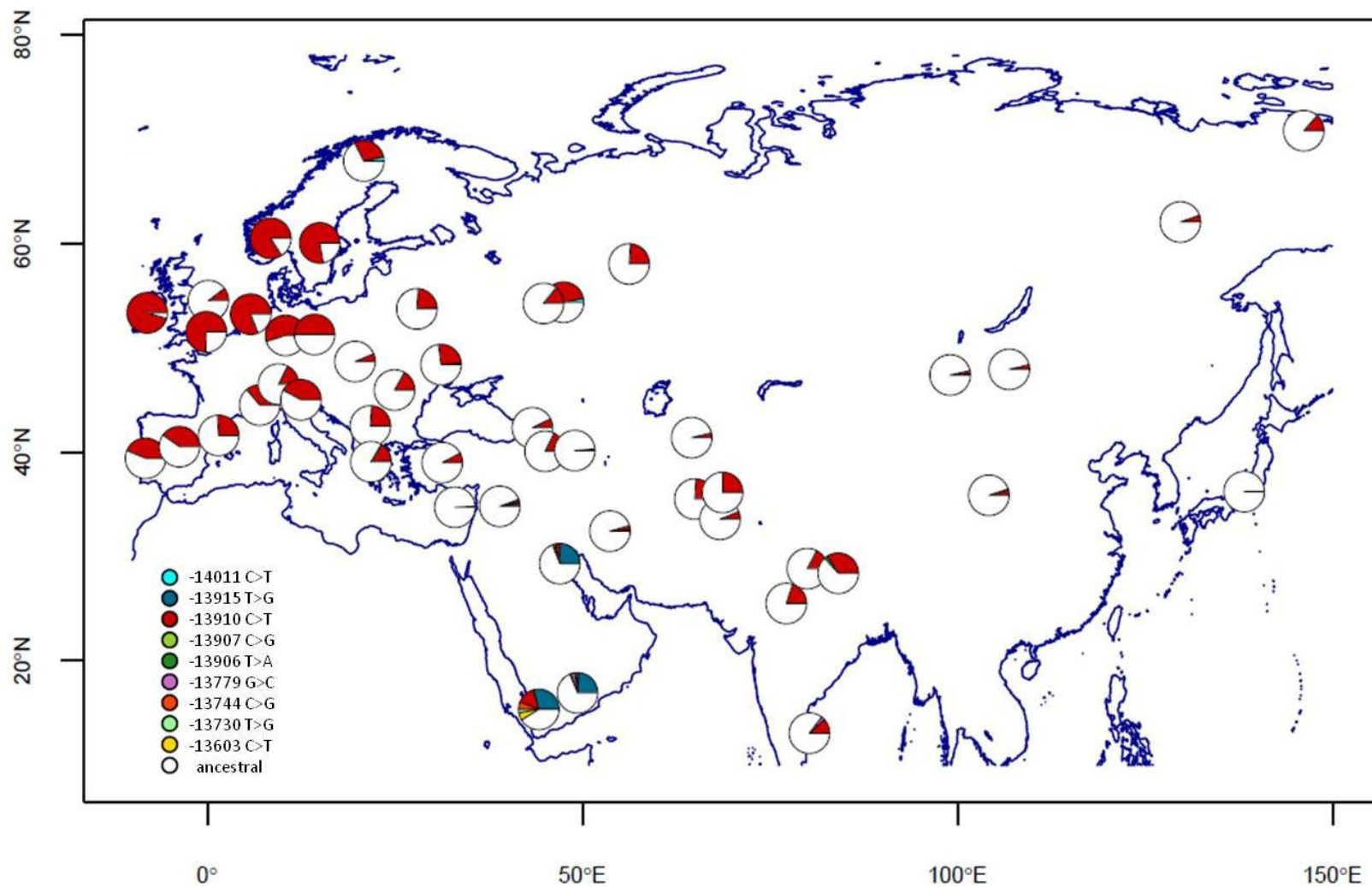


Figure 3.2: Geographic distribution of the common *LCT* enhancer variants (also shown in Table 3.1) in the 52 studied populations (-13495 C>T is not included).

Figure 3.2 shows the geographic distribution of the common alleles within the (likely) functional region of the *LCT* enhancer in the populations studied and illustrates once more the predominance of *-13910*T* as main enhancer variant in the Europe. *-13910*T* was found in all populations tested except of the Japanese (Table 3.1). The results also broadly confirm the decline in allele frequency of *-13910*T* to the South and East of Europe. It is highest in the Irish and Norwegian populations with 96% and 84% respectively and lowest in the Tyroleans from Bozen and in Greeks (both at 15%).

Noticeable different frequencies of *-13910*T* in Northwest and Central Europe are observed in the Sami, the Ashkenazi-Jewish and Roma groups where the frequency of the **T* allele is comparatively low. The Sami are an indigenous population in the far North of Europe and are traditional reindeer herders, subsisting mainly from meat and fishing (Leonard and Crawford 2002). The Ashkenazi-Jewish and Roma have history of migration and were sampled in regions geographically distant from the living places of many of their recent ancestors. The recent Eastern European history of many of the Ashkenazi Jews and their complex history of migrations as close communities accounts for the overall genetic differentiation from the majority of the ethnic English population. The Roma originated in India and are likewise known to form a distinct ethnic-cultural minority mainly in Southeast and Middle Europe.

Differences in *-13910*T* frequencies of very closely located groups appear in the Spanish and the Catalans with a *-13910*T* frequency of 40% and 26% respectively and the Tyroleans between the German and Ladin speaking groups from Vinschgau and Gadertal and the Italian speaking population from Bozen with 36%, 42% and 15% *-13910*T* frequency respectively. The Catalans and Tyroleans are culturally and partly geographically quite isolated populations, which could explain these differences probably caused by decreased gene flow with neighbouring groups. The low *-13910*T* frequency of the Italian speaking Tyroleans, similar to other Italian regions from further South, reflects their recent immigration history into the Alps within the last century (Parteli 1988). The German Tyroleans were shown to be genetically influenced by the Ladins and both being highly genetically differentiated (Pichler et al. 2006).

The SNP -13495 C>T was polymorphic in all populations tested and has previously shown by members of the lab to be is too frequent to be causal of lactase persistence (unpublished data). This variant resides outside the functional enhancer examined by

Troelsen et al. (2003) and it was shown to be non-functional in reporter gene assays as it did not increase activity compared to the ancestral enhancer (Tishkoff et al. 2007).

Table 3.2 shows the distribution of the common enhancer alleles by geographic region. The previously observed pattern of a Northwest-Southeast decline in *-13910*T* frequency (Swallow 2003) is slightly disrupted across Asia. The prevalence of *-13910*T* is higher in Central and South Asia than Western Asia including the Middle East. Populations from Central and South Asia including Afghans, Indians and Nepalese have a similar *-13910*T* frequency to populations from East and Southeast Europe, for example the Macedonians and Belarus. The Georgians and Azeri of the West Asian Caucasus region have very low *-13910*T* frequencies comparable to those of their Middle Eastern neighbours.

Table 3.2: Frequency of the common enhancer alleles by geographic region (-13495 C>T included).

Region	N	-14011 C>T	-13915 T>G	-13910 C>T	-13907 C>G	-13906 T>A	-13779 G>C	-13744 C>G	-13730 T>G	-13603 C>T	-13495 C>T
NW/C Eur.	740	0.003	-	0.615	-	-	-	-	-	-	0.688
S Europe	594	0.003	-	0.305	-	-	-	-	0.002	-	0.359
E/SE Eur.	550	0.005	-	0.231	-	-	-	-	-	-	0.358
Middle East	892	0.001	0.094	0.041	0.003	-	0.007	0.006	0.004	0.006	0.219
W Asia	288	-	-	0.094	-	-	-	-	-	-	0.247
C/S Asia	562	-	-	0.167	-	0.002	0.005	-	-	-	0.370
C/E/SE Asia	480	0.002	-	0.038	-	0.002	-	-	-	-	0.360

*-13945*T* broadly follows the *-13910*T* distribution pattern. It is also particularly high in Northern European populations, even in the Sami as shown in Figure 3.3. To evaluate statistically whether the two alleles correlate with each other and follow the same Northwest to Southeast decline previously suggested for *-13910*T*, in the 48 geographically located populations of this study, Spearman Rank correlation tests were performed. The frequencies of the two markers correlate strongly with each other (Rho = 0.75, $p < 0.01$). A significant negative correlation between *-13910*T* and *-13945*T* and longitude (distance calculated in km from 10°W) was observed which was less strong for *-13945*T* (Rho = -0.39, $p < 0.01$) than for *-13910*T* (Rho = -0.65, $p < 0.01$). The frequency for both alleles also significantly decreases with distance from latitude 80°N (in km). The negative correlation was slightly stronger for *-13945*T* (Rho = -0.52, $p < 0.01$) than for *-13910*T* (Rho = -0.45, $p < 0.01$). It should be noted that these results are consistent with the

finding that -13945*T forms part of the haplotype on which -13910*T arose as described in section 3.6 below.

The other 3 lactase persistence associated alleles (-14010*C, -13915*G and -13907*G) were only found in Middle Eastern populations. -13915*G is at high frequencies in Kuwait (25%) and Yemen (Hadramaut: 24%, Sena: 29%). -14010*C was rare, only occurring once in the Yemen Hadramaut, as well as -13907*G, found in two individuals from Hadramaut and in one other Middle Eastern sample. The two other alleles -14028*C and -13914*A, previously found in Europeans (Ingram 2008; Tag et al. 2008; Tag et al. 2007) occurred in one English individual and in an English person of Ashkenazi-Jewish background respectively.

Noticeable is the occurrence of another allele -14011*T previously described in populations from India, Estonia and Brazil (Friedrich et al. 2012; Gallego Romero et al. 2012; Lember et al. 2006). Although this allele was rare in any of the Eurasian groups tested, it was found scattered across Europe in individuals from Belorussia, Greece, Tyrol, Mordovia, Ukraine, Iran, Mongolia respectively and two Sami individuals and one other individual of mixed ancestry. It additionally occurred in one Saudi Arabian Bedouin sample and one from the phenotyped Italian cohort sequenced for chapter 5.

-13779*C was identified in India, Syria and Yemen. This allele has been reported so far in the literature in one Somali individual who was diagnosed as a lactose maldigester (Ingram et al. 2009b) but is quite frequent in populations from India as shown in our collaborative project (Gallego Romero et al. 2012). Although it might not be associated with lactase persistence in these populations, its frequency in a milk drinking country and its location in the enhancer sequence part that shows a DNase footprint (see chapter 4) could indicate some functional role.

An interesting case is an additional allelic variant at nucleotide position -14010. A Japanese individual carried an A at this position, the same location as the more frequent G>C SNP.

Another new variant is -13744*G, which was found in the Yemeni from Sena (4 individuals) and additionally in one individual of the Yemeni of Hadramaut. It occurs on a haplotype together with the lactase persistence allele -13915*G as shown in section 3.6 below.

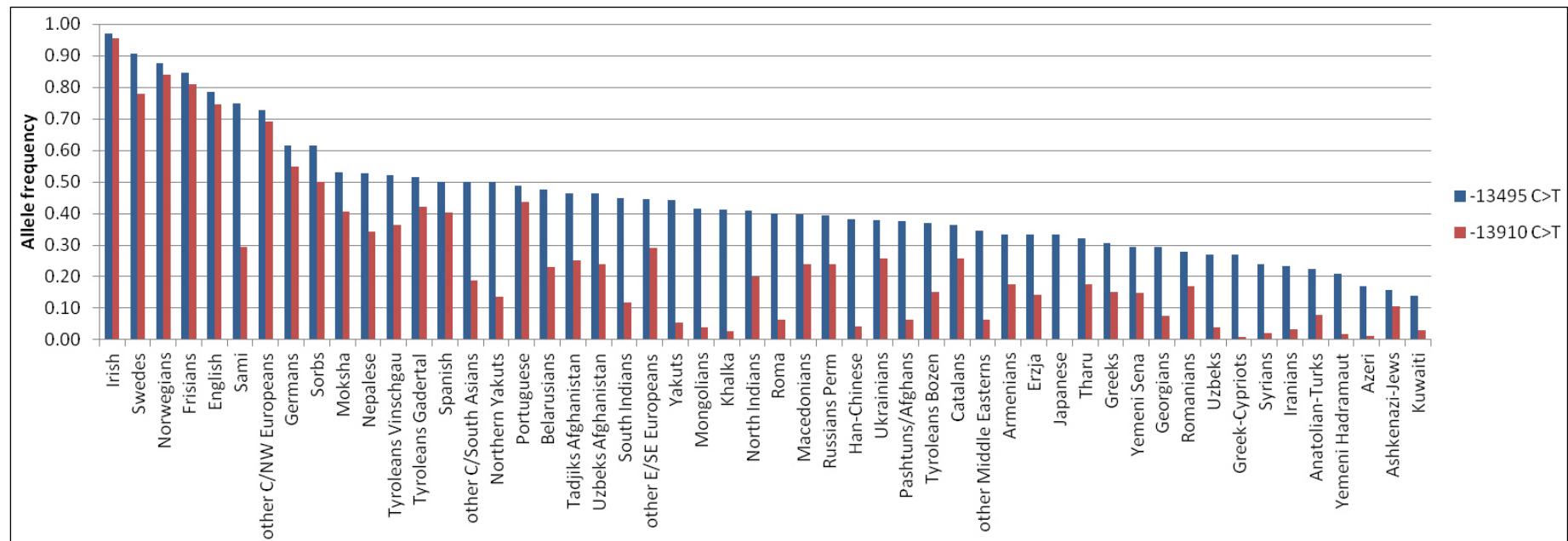


Figure 3.3: Allele frequency of -13945*T and -13910*T for the 52 populations tested, shown in order of -13495*T frequency.

3.6 Relationship of -13495*T to other enhancer alleles

To analyse the relationship of -13495*T to other enhancer alleles haplotype inference was done using PHASE. All samples with incomplete data at the -13945 nucleotide position were excluded which left 1888 samples for analysis. -13495*T occurred on the same haplotypes as four other enhancer variants: -14011*T, 13910*T, -13907*G, -13603*T and was also present on its own as a derived allele on the ancestral enhancer haplotype (Figure 3.4, haplotype no. 2). Chapter 5 will investigate this further in examining -13495*T association with the *LCT* core haplotypes.

Haplotype ID	-14028 T>C	-14011 C>T	-14010 G>C	-14009 T>G	-13915 T>G	-13910 C>T	-13907 C>G	-13906 T>A	-13806 A>G	-13779 G>C	-13744 C>G	-13730 T>G	-13603 C>T	-13495 C>T	N
1	2079
9	T	T	860
2	T	716
10	G	77
5	C	9
15	.	T	T	9
3	T	T	5
4	G	.	.	5
11	G	G	.	.	.	5
7	G	T	3
6	A	2
8	T	2
12	G	1
13	.	.	.	G	1
14	.	.	C	1
16	C	1

Figure 3.4: Haplotype analysis of the *LCT* enhancer region, N: number of chromosomes.

3.7 Population differentiation across the *LCT* enhancer

To compare the genetic distance between the groups, pairwise F_{ST} values were calculated in Arlequin with the enhancer genotype data of the 52 populations studied. Not all populations are significantly different in pairwise F_{ST} when only taking the enhancer region into account (for a full results table, see Appendix D). The -13495 C>T SNP could not be included in this analysis because of too much missing data (9.6%). The F_{ST} values for inter-population differences were used to perform a Principal co-ordinate analysis

(PCO) in R and results are illustrated as PCO plot in Figure 3.5. Over 97% of the population differences are captured in the two first components. The first co-ordinate seems to be highly influenced by -13910^*T as the Northwest European populations cluster on the right half of the plot and all other populations closely together. The Greek Cypriots are the furthest outstanding on the left side as they have the lowest *T allele frequencies and none of the other rare derived enhancer alleles. The two Yemeni populations (51 and 52) can be seen at the upper edge of the point cloud, which may be explained by the numerous other enhancer alleles present in these groups.

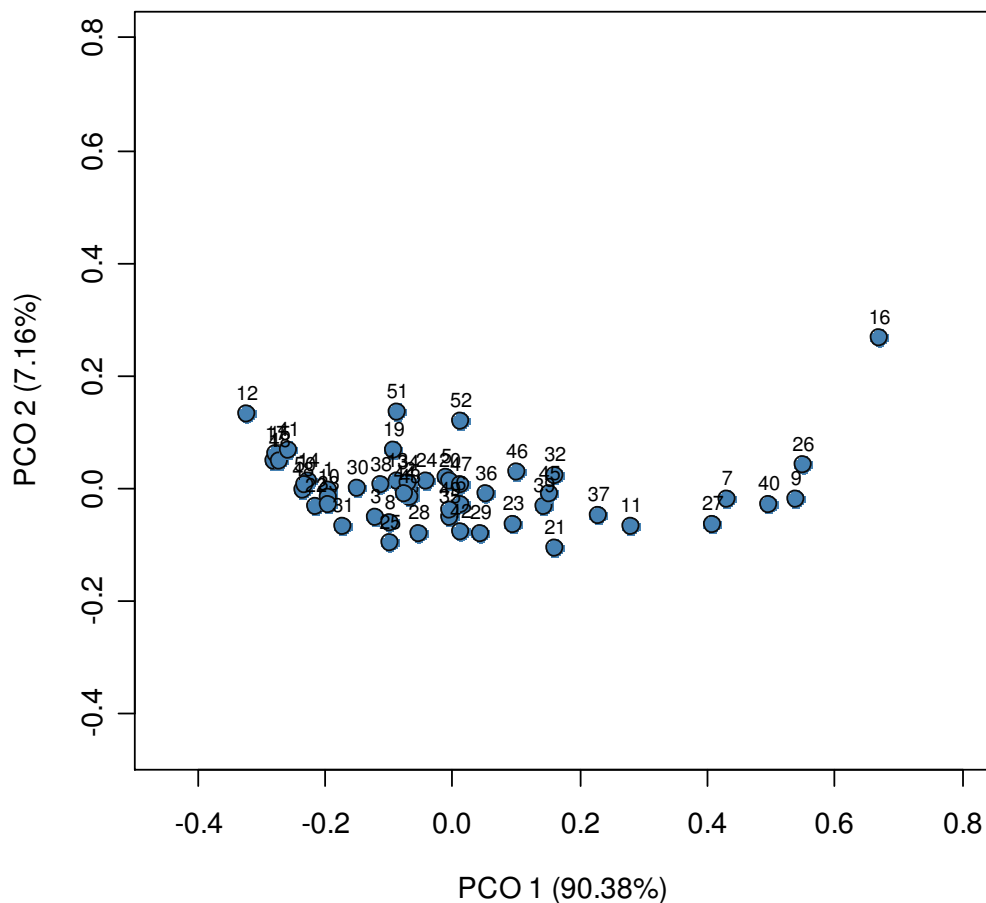


Figure 3.5: Principal co-ordinates plot of genetic distances between populations from pairwise F_{ST} values calculated from LCT enhancer genotype data. Populations included: 1:Anatolian-Turks, 2: Armenians, 3: Ashkenazi-Jews, 4: Azeri, 5: Belarusians, 6: Catalans, 7: English, 8: Erzja, 9: Frisians, 10: Georgians, 11: Germans, 12: Greek Cypriots, 13: Greeks, 14: Han-Chinese, 15: Iranians, 16: Irish, 17: Japanese, 18: Khalka, 19: Kuwaiti, 20: Macedonians, 21: Moksha, 22: Mongolians, 23: Nepalese, 24: North Indians, 25: Northern Yakuts, 26: Norwegians, 27: other C/NW Europeans, 28: other C/South Asians, 29: other E/SE Europeans, 30: other ME, 31: Pashtuns/Afghans, 32: Portuguese, 33: Roma, 34: Romanians, 35: Russians Perm, 36: Sami, 37: Sorbs, 38: South Indians, 39: Spanish, 40: Swedes, 41: Syrians, 42: Tadjiks Afghanistan, 43: Tharu, 44: Tyroleans Bozen, 45: Tyroleans Gadertal, 46: Tyroleans Vinschgau, 47: Ukrainians, 48: Uzbeks, 49: Uzbeks Afghanistan, 50: Yakuts, 51: Yemeni Hadramaut, 52: Yemeni Sena.

3.8 Tests of neutrality and diversity measures

Haplotypes of the enhancer region as inferred by PHASE were used with the DnaSP software to calculate haplotype and nucleotide diversity for each population. Several neutrality tests were also conducted to detect a possible excess of rare alleles, which might signify a selective sweep. Table 3.3 shows the values calculated for each population. The only significant deviation from neutrality was seen for Tajima's *D* in the case of the German and Ladin speaking groups and the Portuguese and Spanish. These showed a positive value, which is usually taken as a sign of balancing selection (alleles of equal frequency). However when excluding the -13495 C>T SNP from analysis none of them remained significant (data not shown).

3.9 Lactase persistence genotype-phenotype correlation

The frequencies of the lactase persistence associated alleles in the populations tested from Europe and Asia give an idea about how the lactase persistence phenotype is distributed. To see whether the predicted lactase persistence frequency from genotype data for the populations examined fit with observed published phenotype frequencies a GenoPheno test (Mulcare et al. 2004) was applied (for details see 1.3.10). Phenotype data from matching population samples were extracted from the 'Global Lactase persistence Association Database' (GLAD, <http://www.ucl.ac.uk/mace-lab/resources/glad>) compiled from different sources (Itan et al. 2010). Groups were selected as matching when they were from the same self-declared cultural identity living in the same or a directly neighbouring country.

Table 3.4 shows that for most of the groups studied, the frequencies of lactase persistence predicted from genotype data correlates well with published phenotype data. Significant differences were however observed in the Roma, English, Greek Cypriots and in Asia in the Uzbeks from Afghanistan, North Indians and Japanese, where the predicted frequencies were either higher or lower than those that were reported from phenotypic testing. However, taking multiple testing into account (22 tests) only the Uzbeks, North Indians, and Greek Cypriots remain significant, with the more stringent *p*-value threshold of 0.002. These differences may in some cases result from sampling of slightly different populations for genotype and phenotype data and in others may reflect as yet undiscovered persistence alleles.

Table 3.3: Diversity and neutrality measures for all 52 tested populations. Significant values ($p < 0.05$) are shaded, N: number of chromosomes.

Region	Population	N	Haplotype diversity (Nei's H) (\pm SD)	Nucleotide diversity per site (π)	Tajima's D	Fu and Li's D^*	Fu and Li's F^*
Northwest/ Central Europe	Germans	60	0.555 (0.001)	0.002	2.213	0.729	1.365
	Sorbs	64	0.603 (0.001)	0.002	2.235	0.723	1.370
	Irish	68	0.086 (0.002)	0.000	-1.091	0.718	0.201
	Frisians	58	0.324 (0.005)	0.001	0.588	0.733	0.802
	Norwegians	88	0.279 (0.003)	0.001	0.381	0.696	0.700
	Roma	64	0.482 (0.003)	0.001	0.574	0.723	0.790
	Sami	60	0.689 (0.001)	0.002	0.762	0.873	0.979
	Swedes	74	0.383 (0.004)	0.001	0.816	0.710	0.865
	Ashkenazi-Jews	38	0.284 (0.008)	0.001	-0.038	0.777	0.627
	English	102	0.405 (0.003)	0.001	0.519	-0.590	-0.282
South Europe	other C/NW Europeans	68	0.450 (0.003)	0.002	1.690	0.718	1.177
	Greeks	120	0.474 (0.002)	0.001	-0.101	-1.534	-1.259
	Tyroleans Bozen	80	0.526 (0.003)	0.001	0.427	-0.534	-0.274
	Tyroleans Gadertal	76	0.585 (0.001)	0.002	2.357	0.708	1.409
	Tyroleans Vinschgau	102	0.613 (0.001)	0.002	2.336	0.684	1.396
	Portuguese	96	0.561 (0.000)	0.002	2.440	0.689	1.435
	Catalans	58	0.525 (0.003)	0.002	1.707	0.733	1.190
	Spanish	62	0.559 (0.001)	0.002	2.268	0.726	1.383
	Belarusians	100	0.636 (0.001)	0.002	0.940	-0.586	-0.123
	Macedonians	100	0.553 (0.002)	0.002	1.842	0.685	1.219
Southeast Europe	Romanians	118	0.444 (0.002)	0.001	1.259	0.673	1.002
	Erzya	42	0.511 (0.005)	0.001	0.968	0.766	0.956
	Moksha	32	0.625 (0.002)	0.002	1.023	-0.283	0.110
	Russians Perm	46	0.590 (0.004)	0.002	1.616	0.756	1.170
	Ukrainians	74	0.529 (0.003)	0.002	0.828	-0.515	-0.115
	Other E/SE Europeans	38	0.602 (0.003)	0.002	1.827	0.777	1.251
Middle East	Greek Cypriots	120	0.365 (0.002)	0.001	0.017	-1.113	-0.892
	Iranians	154	0.386 (0.002)	0.001	-0.682	-1.617	-1.548
	Kuwaiti	66	0.591 (0.003)	0.001	-0.953	-1.549	-1.595
	Syrians	140	0.402 (0.002)	0.001	-0.708	-0.337	-0.541
	Anatolian Turks	116	0.340 (0.003)	0.001	0.357	0.674	0.674
	Yemeni Hadramaut	166	0.661 (0.001)	0.002	-1.059	-1.943	-1.942
	Yemeni Sena	68	0.773 (0.001)	0.002	0.244	1.151	1.008
	Other Middle Easterns	62	0.548 (0.003)	0.001	-0.405	-0.153	-0.271
	Armenians	102	0.505 (0.002)	0.001	1.423	0.684	1.067
	Azeri	80	0.279 (0.003)	0.001	-0.411	-1.017	-0.971
Central/ South Asia	Georgians	108	0.394 (0.003)	0.001	0.505	0.680	0.732
	Pashtuns/Afghans	32	0.524 (0.004)	0.001	0.438	0.798	0.804
	Tadjiks Afghanistan	28	0.627 (0.004)	0.002	1.591	0.815	1.192
	Uzbeks Afghanistan	54	0.616 (0.002)	0.002	1.757	0.740	1.210
	North Indians	120	0.511 (0.002)	0.001	1.607	0.672	1.128
	South Indians	102	0.606 (0.001)	0.001	0.583	0.825	0.878
	Nepalese	38	0.666 (0.002)	0.002	0.968	-0.335	0.056
	Tharu	80	0.484 (0.003)	0.001	1.288	0.704	1.029
	Uzbeks	76	0.334 (0.004)	0.001	-0.040	0.708	0.561
	Other C/South Asians	32	0.605 (0.004)	0.001	1.319	0.798	1.093
Central/East/ Southeast Asia	Khalka	114	0.499 (0.001)	0.001	-0.068	-0.615	-0.519
	Mongols	52	0.520 (0.002)	0.001	0.494	0.743	0.778
	Northern Yakuts	22	0.619 (0.006)	0.001	0.845	0.851	0.976
	Yakuts	110	0.535 (0.001)	0.001	0.871	0.678	0.864
	Han-Chinese	98	0.456 (0.002)	0.001	-0.190	-0.581	-0.537
	Japanese	82	0.438 (0.001)	0.001	1.413	0.505	0.901

Table 3.4: GenoPheno analysis of studied populations where published lactase persistence or lactose digester frequency data were available.
Significantly different values are marked in bold, significant values after Bonferroni correction are shaded.

Population	No. of chromosomes	Frequency LP associated alleles	No. of individuals	reported LP frequency	GenoPheno <i>p</i> -value	Reference published data
Germans	60	0.550	221	0.864	0.094	Flatz, G., et al. (1982) Hum.Genet 62, 152.
Irish	68	0.956	50	0.960	0.553	Fielding et al. (1981) Ir J Med Sci. 150, 276.
Roma	64	0.063	113	0.442	0.003	Czeizel et al. (1983) HumGenet. 64, 398.
Sami	60	0.293	50	0.520	1.000	Kozlov (1998) Int J Circumpolar Health. 57, 18.
Swedes	74	0.778	156	0.827	0.311	Sahi (1974) Scand J Gastroenterol. 9, 303.
English	102	0.745	150	0.953	0.020	Ferguson et al. (1984) Gut. 25, 163.
Greeks	120	0.150	200	0.553	0.226	Kanaghinis et al. (1974) Am J Dig Dis. 19, 1021.
Spanish	62	0.403	338	0.660	0.806	Leis et al. (1997) J Pediatr Gastroenterol Nutr. 25, 296.
Russians Perm	46	0.239	112	0.500	0.629	Kozlov (1998) Int J Circumpolar Health. 57, 18.
Greek Cypriots	120	0.008	50	0.340	0.002	Kanaghinis et al. (1974) Am J Dig Dis. 19, 1021.
Iranians	154	0.039	21	0.143	1.000	Sadre et al. (1979) Am J Clin Nutr. 32, 1948.
Kuwaiti	66	0.295	70	0.529	0.920	Sanae et al. (2003) Med Princ Pract. 12, 160.
Anatolian-Turks	116	0.078	104	0.288	0.271	Flatz et al. (1986) Am J Hum Genet. 38, 515.
Yemeni Hadramaut	166	0.277	17	0.530	0.972	Dissanayake et al. (1990) Ann Saudi Med. 10, 598.
Yemeni Sena	68	0.441	17	0.530	0.357	Dissanayake et al. (1990) Ann Saudi Med. 10, 598.
Pashtuns/Afghans	32	0.063	71	0.211	0.971	Rahimi et al. (1976) Hum Genet. 34, 57.
Tadjiks Afghanistan	28	0.250	79	0.177	0.006	Rahimi et al. (1976) Hum Genet. 34, 57.
Uzbeks Afghanistan	54	0.241	16	0.000	0.001	Rahimi et al. (1976) Hum Genet. 34, 57.
North Indians	120	0.200	70	0.729	0.000	Gupta et al. (1971) J Trop Med Hyg. 74, 225.
South Indians	102	0.118	100	0.360	0.309	Desai et al. (1970) Indian J Med Sci. 24, 729.
Han-Chinese	98	0.041	248	0.077	0.052	Yongfa et al. (1984) Hum Genet. 67, 103.
Japanese	84	0.000	40	0.275	0.028	Yoshida et al. (1975) Gastroenterol Jpn. 10, 29.

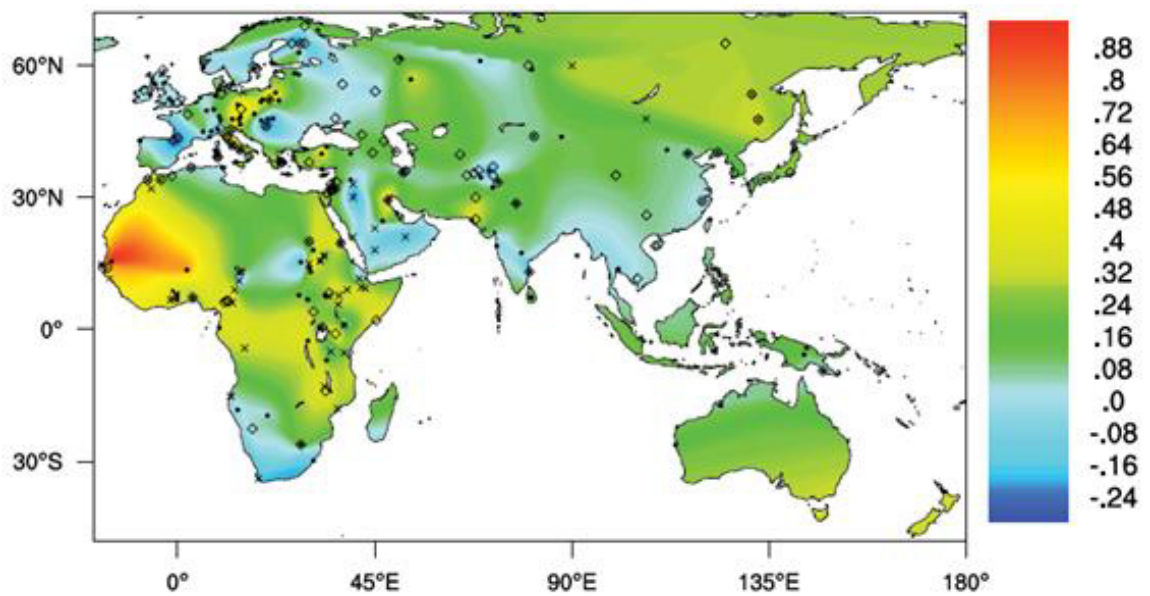
3.9.1 Worldwide genotype-phenotype correlation

Itan et al. (2010) devised a method for comparing lactase persistence genotype and phenotype data using interpolated values for regions with data lacking-one or the other type of information. Since the original publication of this approach in 2010, more publications giving genotype data for the *LCT* enhancer have become available and very recently a variant with previously reported borderline association, *-14009*G* (Ingram et al. 2009b), was shown by our group to be significantly associated with lactase persistence in a larger Ethiopians cohort (Jones et al. 2013).

A new interpolated lactase persistence genotype-phenotype correlation map of the Old World was constructed, with the help of Pascale Gerbault and Yuval Itan using the same Python code as in the original publication (Itan et al. 2010), to see whether the data collected in this thesis, the additional lactase persistence associated allele and the newly published data change the association patterns of the map. A literature review was necessary to update the GLAD database used for the original publication (Itan et al. 2010), this also included correction of coordinates of some data collection points. Tables with the full updated genotype and phenotype data are shown in Appendix B. Figure 3.6 and Figure 3.7 show the new lactase persistence genotype-phenotype correlation maps in comparison with the originally published maps by Itan et al. (2010).

The new data has contributed considerably to the density of genotype data points across Europe, South Asia and East Africa. The new genotype data for Oman (Al-Abri et al. 2012) has added to the areas where the lactase persistence phenotype was overpredicted by genetics. Areas where observed lactase persistence frequencies were not fully predicted from genotype data were slightly reduced but could not completely be resolved, such as in West Africa, the centre of Italy, some Eastern European areas and South Asian areas around Pakistan and North India. However, information about lactase persistence phenotype and genotype data is still rare in North and East Asia, the North and other parts of Africa and Australia and South East Asia

a)



b)

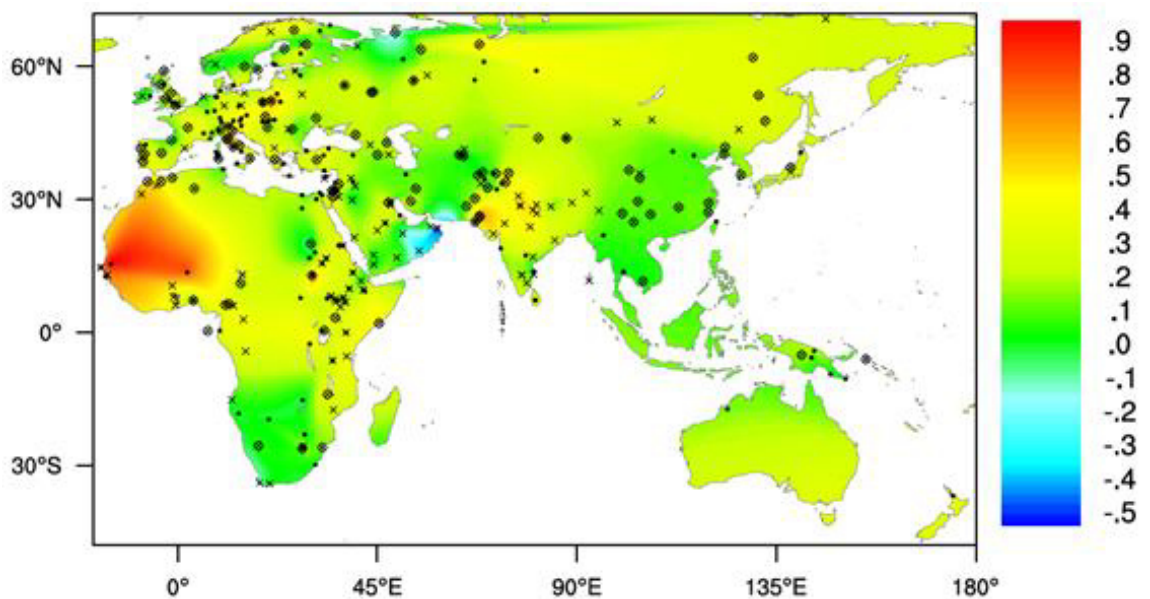
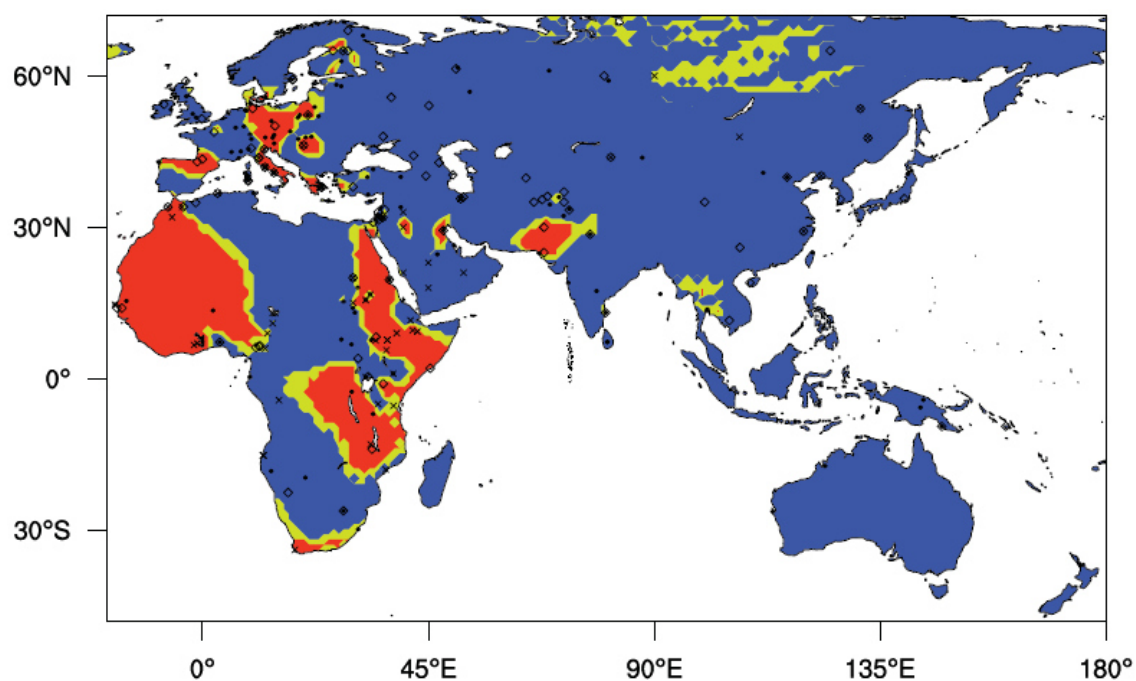


Figure 3.6: LP genotype-phenotype subtractive map of the Old World. The map shows quantitative differences between observed LP phenotype data and these predicted from genotype data (both interpolated). Allele frequencies of the alleles *-14010*C*, *-14009*G*, *-13915*G*, *-13910*T* and *-13907*G* were taken into account to predict LP phenotype frequencies, which were subtracted from the observed LP phenotype frequencies. For geographic regions where no phenotype or genotype data were available, values were interpolated. a) shows the map originally published in Itan et al. (2010) and b) the newly calculated map. Colour key on the right: + values represent the genotype under-predicting phenotype at a certain location and - values over-predicting it, respectively. Note that the colour codes as shown in the scales on the right differ slightly between the maps, because of the greater range in the second map. Data collection points: \diamond -*-13910*T*, x all 4 or 5 LP alleles, \bullet LP phenotype.

a)



b)

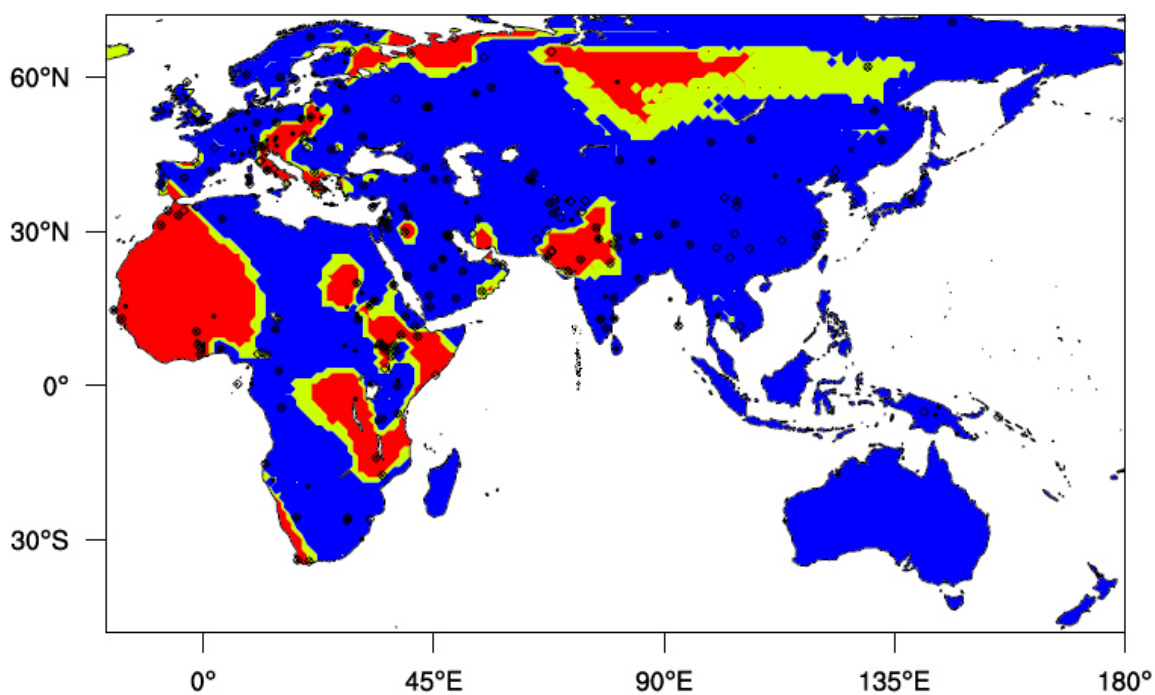


Figure 3.7: P -value contour map corresponding to the LP genotype-phenotype subtractive map of the Old World, shown in Figure 3.6. The colours represent the p -values obtained by the GenoPheno test: A highly significant difference is shown in red ($p < 0.01$), yellow shows p -values of $0.01 \leq p \leq 0.05$ and blue indicates $p \geq 0.05$. Data collection points: \diamond -13910*T only, x all 4 or 5 LP alleles, \bullet LP phenotype.

3.10 Discussion

At the outset of this project European and Asian populations had mainly been typed specifically for the -13910 C>T SNP and only a few studies provided full enhancer sequencing data. Although more data are now available (see Appendix B for a full literature survey), this study represents one of the widest geographic surveys to date of the *LCT* enhancer sequence variation across Eurasia obtained via sequencing.

The results show that -13910*T is present in all populations tested except the Japanese, even in the Middle East at low frequencies. The allele is most prevalent in Northwest Europe and declines in frequency to the South and East as previously shown (Swallow 2003). However, deviating from this general pattern some of the Central and South Asian populations show similar -13910*T frequencies to Southern Europeans.

The other lactase persistence associated alleles on the other hand were only found in the Middle Eastern populations and of these mainly -13915*G, the others being surprisingly rare. -14010*T only occurred as a singleton as did -14009*G.

In contrast to this -13495*T was present in all populations at considerable frequencies. It broadly follows the Northwest to Southeast frequency decline of -13910*T, which could be explained by the fact that it occurs on the same haplotype background as -13910*T. It also occurs in combination with -13907*G and -14011*T, which might suggest it to be an evolutionary older variant. It was previously reported on a much smaller dataset to be in strong LD with -13910*T and -13907*G (Ingram 2008) and in association with the A haplotype but it was too frequent to be causal of lactase persistence. Further study of its pattern of association with the classical *LCT* haplotype markers in a wider-ranging set of populations, including some African groups should give better insight into the evolutionary relationship of this allele to the known haplotypes and is described in chapter 5.

Most of the newly discovered variants are rare and only one case of each of -14028*C and -13914*A were present in the European data. -13914*A was recently reported in one individual with high lactase activity in biopsy material (Khabarova et al. 2010) which did not carry other enhancer alleles thus could be, like 14028*C, a candidate for causing lactase persistence.

-14011*T is rare-as well, but can be found in various European populations, which is of interest. Further study of the haplotype background of this allele might give insight into its origins, and this is also described in chapter 5.

None of the novel alleles occurred at frequencies high enough in any particular population to make new association studies a sensible option, but several of the alleles could be tested for function *in vitro* (chapter 4).

Several tests were conducted to investigate possible signs of selection in the enhancer region of the populations studied. Diversity measures as haplotype and nucleotide diversity as well as test for deviation from neutrality did not reveal any results that pointed to positive selection. It is most likely that the 559 bp enhancer region is too short to detect selection with these methods. The only clear genetic differentiation of the populations, as measured by pairwise F_{ST} , highlighted Northwestern Europeans as different, most probably because of their high -13910*T frequency.

Previously collected lactase persistence data, which was updated with new published information, and genetic data collected for this chapter were used to construct new interpolated maps showing the difference between reported lactase persistence and lactase persistence predicted from genotype, and of statistical significance of these differences calculated by the GenoPheno approach. Compared with the original publication (Itan et al. 2010) new data considerably increase the density of the data points and therefore the quality of the information that can be obtained by such an approach. However it did not decrease the areas with largest discrepancies nor change very much. The weaknesses of the interpolated GenoPheno approach were discussed extensively in Itan et al. (2010). The main issue in the case of lactase seems to be sampling from neighbouring populations with completely different lactase persistence frequencies. The interpolation approach is nevertheless a powerful tool to compare geno- and phenotypic information where it is not possible to collect both in the same populations, and the more data there are the more the effect of heterogeneity is reduced.

The new sequence data obtained in thesis has added considerably to the knowledge of the lactase persistence associated alleles in Eastern Europe and Asia. It was also shown that lacking information for lactase persistence enhancer alleles other than -13910*T was not the reason for the under-predicted phenotype from genotype data in the areas studied. There are still parts of East and South Europe and Asia, namely the regions

of West Russia, Italy and Pakistan and parts of Asia, where genotype data cannot explain the frequency of lactase persistence, which leads to speculations about other genetic influences on the lactase persistence trait. It is possible that further alleles have a functional effect. The next chapter will look further at some of the promising candidate alleles as they were tested in *in vitro* assays.

4 Functional studies of enhancer variation

4.1 Introduction

As discussed in chapter 3, a number of variants have by now been described within the sequence shown by Troelsen et al. (for example 2003) to have enhancer function. Several of these have been subjected to functional studies and showed differences in protein binding affinity and/or expression levels. These variants and the transcription factor binding sites identified so far are shown in Figure 4.1.

Chr 2: 136.608,420-136.608,869 (GRCh37/hg19 reference sequence)
(reverse complement)

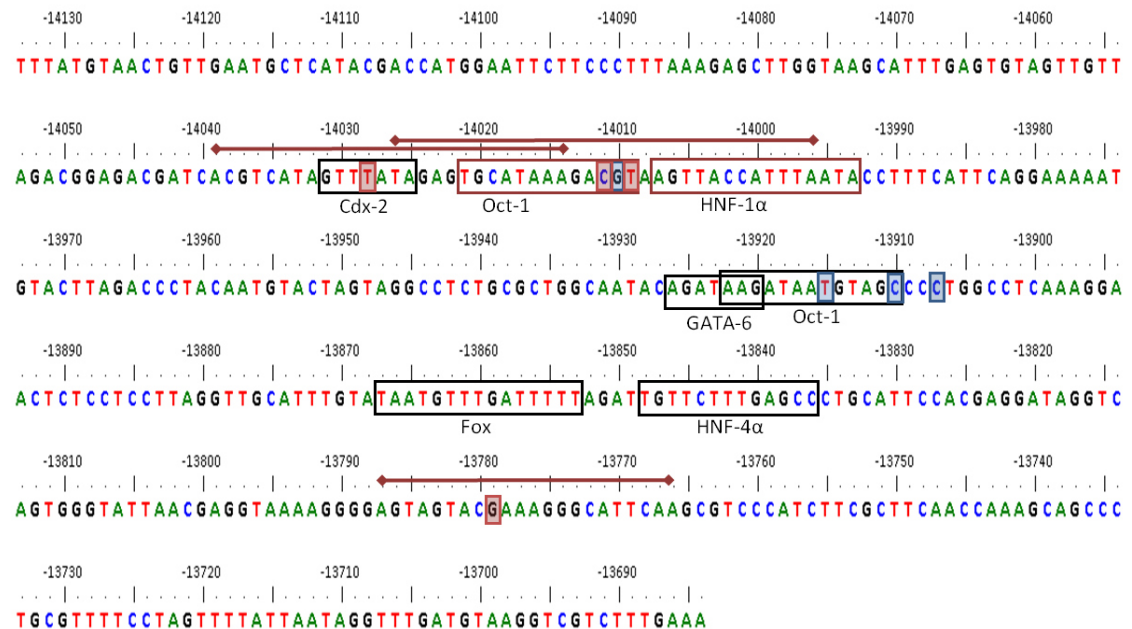


Figure 4.1: Positions in the 450 bp *LCT* enhancer of transcription factor binding sites and SNPs studied functionally. Indicated in boxes: black: transcription factor binding sites as shown in experiments before the outset of this thesis (Lewinsky et al. 2005); red: transcription factor binding sites as shown in experiments during the time of this thesis (Jensen et al. 2011); blue shadowed: SNPs previously examined functionally (Enattah et al. 2008; Ingram et al. 2007; Olds et al. 2011; Tishkoff et al. 2007); red shadowed: SNPs examined functionally within this thesis. EMSA probes used in this thesis are indicated with red lines.

The ‘European’ allele *-13910*T* was the first proposed to influence the level of enzymatic activity and to prevent the post-weaning decline of lactase, by preventing down-regulation of transcription, with evidence from reporter gene experiments and with the findings of increased Oct-1 transcription factor binding in EMSA studies (Ingram et al. 2007;

Lewinsky et al. 2005; Olds and Sibley 2003; Troelsen et al. 2003) (see also chapter 1). The causal role of *-13910*T* for lactase persistence is now even more evident as *in vitro* data were supported by the demonstration of its function in mice (Fang et al. 2012). Fang and colleagues established three transgenic mouse lines carrying a pGL3 luciferase reporter gene plasmid DNA, which included 218 bp of the *LCT* enhancer with either *-13910*T* or **C*, fused to a 2 kb rat *LCT* promoter. As demonstrated previously (Lee et al. 2002) the construct carrying the ancestral variant was able to create the typical lactase spatial and temporal expression pattern in mice, shown by *in vivo* bioluminescent detection and luciferase expression measurements. The two other mouse lines carrying different copy numbers of the *-13910*T* construct showed continued expression into adulthood (Fang et al. 2012).

Several other alleles in close proximity to *-13910*T* have also been studied since their location in and near the same Oct-1 binding site (see Figure 4.1) suggested a similar functional effect.

The lactase persistence associated alleles *-13915*G* and *-13907*G*, as well as *-13913*C*, which has only recently been shown to not be associated with lactose digester status (Jones et al. 2013), were tested in gel shift assays (Enattah et al. 2008; Ingram et al. 2007; Olds et al. 2011). The results of various investigators were slightly different. Compared with *-13910*T*, only minor Oct-1 protein binding of oligonucleotides carrying *-13907*G* was detected by Ingram et al. (2007), and was similar to that of the ancestral allele, whereas no binding at all was seen for *-13915*G* and *-13913*C*. Enattah and colleagues (2008) also showed no or very weak binding to *-13915*G* and *-13913*C* but their experiments revealed Oct-1 binding to the *-13907*G* probe at a level similar to *-13910*T*. Yet another study claimed to show binding of *-13915*G* to proteins of the nuclear extract, identified as partly being Oct-1 with competition and supershift experiments (Olds et al. 2011).

Transcription factor binding capacity *in vitro* is clearly complex so that gel shifts assays only give hints of differences between the alleles but results of transfection experiments provide further functional evidence. *-13915*G* and *-13907*G* alleles were shown clearly by several groups to increase reporter gene expression compared to the ancestral constructs (Enattah et al. 2008; Olds et al. 2011; Tishkoff et al. 2007). The Tishkoff study also included the lactase persistence associated allele *-14010*C*, which was shown to drive a higher *LCT* promoter expression too (Tishkoff et al. 2007). However, the influence of

transcription factor binding of the -14010 G>C SNP had only been tested in preliminary experiments at the outset of this thesis.

The -14010*C variant was further examined in a project I was involved in, lead by Tine Jensen of the Troelsen group in Copenhagen. It was shown that the derived allele at -14010 G>C increased reporter gene expression compared to the ancestral variant. Gel shift assays located the SNP between an Oct-1 and HNF-1 α transcription factor binding site and stronger binding of -14010*C to Oct-1 was reported (Jensen et al. 2011), see Appendix G. The functional region of the 450 bp enhancer described previously (Lewinsky et al. 2005; Troelsen et al. 2003) was investigated once more and it was shown that the 5' part of the enhancer region (-14133 to -13990) negatively influenced enhancer function, since the deletion of this part increased the activity of the remaining enhancer.

4.2 Choice of candidate functional variants

The focus in chapter 3 was on the search for new *LCT* enhancer variant alleles in European, Middle Eastern and other Asian populations that could tentatively be involved in lactase persistence and which would be further studied *in vitro*. Although some new alleles were found, they are quite rare, so it was not possible as a consequence to collect phenotypic data to test for association. Therefore four variants (-14009*G, -14011*T, -14028*C, -13779*G) were instead chosen to conduct *in vitro* functional assays, after a review of other studies in combination with the information in chapter 3.

-14009*G

-14009*G was found only once in the samples studied in chapter 3. However the association of -14009*G with lactase persistence had previously been shown to be of borderline significance in the Somali of Ethiopia (Ingram et al. 2009b). This allele is quite common in Ethiopia and Sudan and was noteworthy for being adjacent to a known lactase persistence variant, -14010*C. As described in the previous chapter, -14009*G has recently been confirmed to be associated with lactose digester status in a larger Ethiopian cohort (Jones et al. 2013).

-14011*T

-14011*T is rare but appears in several Eurasian groups as well as in a populations of Iran, Ethiopia and Brazil (Friedrich et al. 2012; Jones et al. 2013; Lember et al. 2006 and several groups in chapter 3 of this thesis) and like -14009*G is adjacent to a reported lactase persistence allele, which lead to speculations about possible function.

-14028*C

Another particularly interesting variant, -14028*C, was found in a European individual with two high lactase expressing alleles but only one chromosome containing -13910*T (Poulter et al. 2003), and a further individual of English descent in this study. Lewinsky et al. (2005) had found a Cdx-2 binding site overlapping with this position (see Figure 4.1), which was another reason to investigate the influence of this SNP more in detail.

-13779*G

In the present study a further allele, -13779*G, was found in India, Syria and Yemen (see chapter 3) and it was quite frequent in other populations from India as concurrently shown in a collaborative project (Gallego Romero et al. 2012). The preliminary suggestion that its frequency was higher in certain pastoralist than non-pastoralist Indian groups (Gallego Romero, personal communication 2009) motivated the choice of this allele but in fact this could not be confirmed with the final data of the paper (Gallego Romero et al. 2012). However, additional evidence made this SNP interesting to examine.

Figure 4.2 shows a photograph of a DNase I footprint analysis of the *LCT* enhancer. Around nucleotide position -13800 a footprint of an unknown factor can be seen, suggesting some transcription factor binding there, which could be influenced by the -13779 C>G SNP.

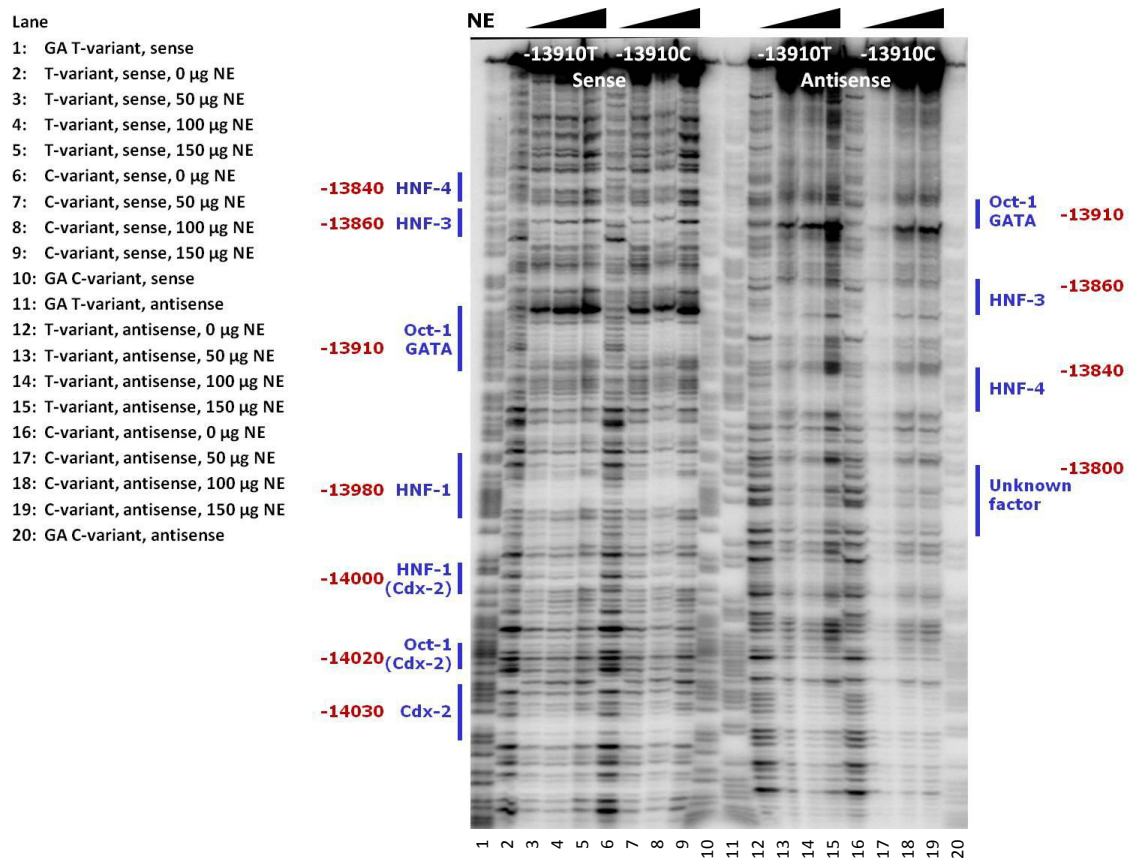


Figure 4.2: Autoradiograph picture of a DNase I footprint analysis done by denaturing polyacrylamide gel electrophoresis (PAGE), kindly provided by Jesper Troelsen. Blue bars indicate footprints with already identified transcription factor binding sites or unknown factors binding. Bp positions upstream of the transcription start of *LCT* are shown in red. With an increase of nuclear extract (NE) footprints get visible as light shaded areas, caused by binding of NE-proteins to the DNA and inhibition of DNase I cleavage.

4.3 Chapter aims

In summary, four *LCT* enhancer variants, -14028*C, -13779*G, -14011*T and -14009*G were selected for *in vitro* functional studies. These would be tested in comparison with the ancestral alleles and the lactase persistence alleles -13910*T and -14010*C. The combination of results from electrophoretic mobility shift essays and luciferase reporter gene assays should provide evidence as to whether these alleles are likely to be involved in the alteration of function of the *LCT* enhancer.

This chapter aims to investigate the influence of the four chosen allelic variants bioinformatically and *in vitro* by:

- Predicting transcription factors binding to the variants and the surrounding DNA regions, especially differences that occur for each allele of a SNP, by using different software tools,
- Conducting EMSA experiments to evaluate differences in binding of proteins of the nuclear extract to DNA sequences containing the variants and test specifically for binding of the predicted transcription factors,
- Transfecting Caco-2 cells with mutated plasmid DNA and measuring differences in expression of a reporter gene.

All experiments were conducted in collaboration with Jesper Troelsen and colleagues from Roskilde University and the University of Copenhagen in Denmark.

4.4 Prediction of transcription factor binding

A first step before conducting functional experiments was to analyse *in silico* the genetic region including 10 bp either side of a SNP or the full length sequence of the designed oligonucleotides for possible transcription factors binding. This was done by using different programs to compare these sequences with matrices of preferred targets for transcription factor binding as curated from literature (for more details see chapter 2, section 2.3.13). An important aspect was the matrix specificity to vertebrate species as considered in MATCH and TFSEARCH, both using the TRANSFAC database but with slightly different statistical algorithms and MatInspector.

As expected, the predicted results overlap to a great extent between the three programs, results are shown in the summary table in Appendix E. Candidate transcription factors

were those with different matrix matches/scores for the ancestral and derived variant and oligonucleotides were chosen to be tested in EMSAs if associated with expression in the digestive system (information from literature or MatInspector) and/or Caco-2 cells (information from 'The intestinal transcription factor target database' of the Copenhagen University, <http://gastro.sund.ku.dk/chipchip/>, with Caco-2 expression data from GeneChip analysis). As time for the experiments was limited, the transcription factors known to be involved in lactase expression, such as Cdx-2, Oct-1 and HNF-1 α were tested first.

Matching competitor oligonucleotides were designed using information about binding site motifs from of the TRANSFAC database (see Table 4.1 below). For some of the predicted transcription factors, information about matching competitor oligonucleotides was available from the Affymetrix EMSA kit and these used to prepare oligonucleotides for competition experiments as described in chapter 2.

4.5 Transcription factor binding affinity of *LCT* enhancer variants

EMSAs were performed as described in section 2.2.6. For all *LCT* enhancer variant probes examined, binding specificity to proteins of the Caco-2 nuclear extract was confirmed if the DNA-protein complex could be competed with an excess of unlabelled oligonucleotides with the same sequences. The effect of competitor oligonucleotides for Cdx-2, Oct-1 and HNF-1 α was in most cases tested, since these TFs have been shown to play an important role in the function of the *LCT* enhancer, as well as other specific probes selected from the *in silico* predictions. By adding target antibodies and specific competitors with a confirmed transcription factor binding sequence, several different factors were identified to bind to the variant probes (as summarised in Table 4.2 and Appendix E).

4.5.1 The influence of -14028 T>C on transcription factor binding

Both alleles of the -14028 T>C SNP bind similarly strongly to proteins of the nuclear extract. This binding could in both cases be competed with unlabelled oligonucleotides containing Oct-1 and HNF-1 α binding sites (not shown). The binding of Oct-1 was expected as the probe sequence overlaps 3' with an Oct-1 site recently reported (Jensen et al. 2011)(see also Figure 4.1). The competition with the HNF-1 α competitor suggests that this is probably another factor involved in the function of the -14133 to -14020 gene-regulatory region, which has been shown to decrease enhancer function in reporter gene

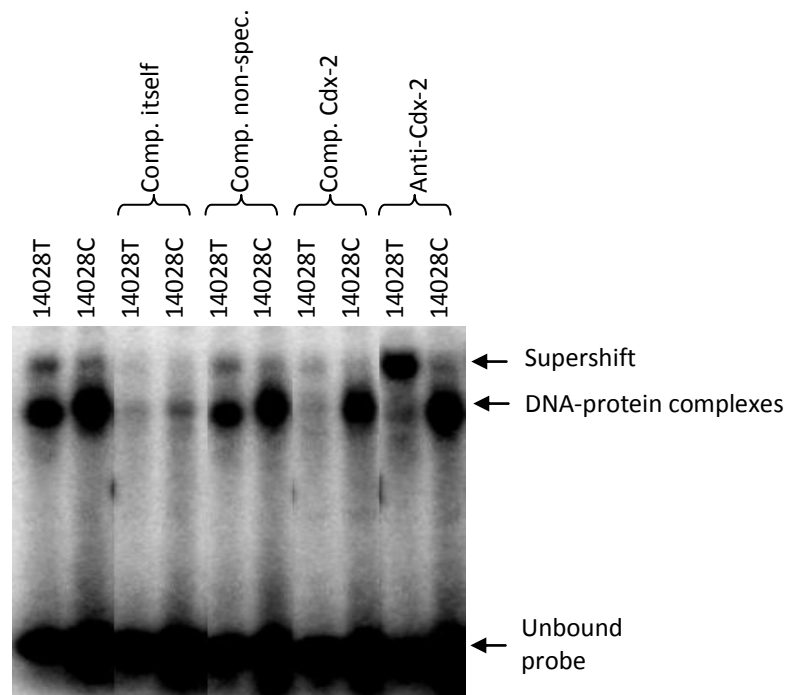
assays (Jensen et al. 2011). However, binding of these transcription factors could not be shown in supershift experiments.

The location of the -14028 position in a Cdx-2 binding site as proposed by Lewinsky et al. (2005) was confirmed. Both allelic probes of the SNP were competed with the same unlabelled Cdx-2 oligonucleotide but the ancestral variant much more strongly than the derived one. A clear supershift could be seen for 14028T with a Cdx-2 antibody (Figure 4.3a).

DNA-protein bands with 14028C were decreased with the addition of an HNF-4 α competitor (Figure 4.3b). A supershift with the HNF-4 α antibody confirmed that this transcription factor is binding to the 14028C probe.

-14028 T>C

a)



b)

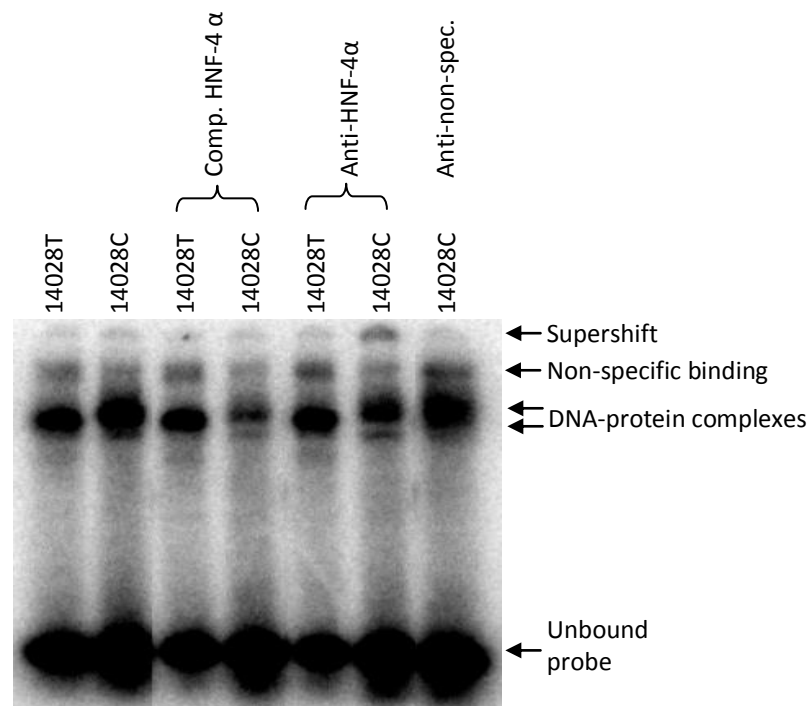


Figure 4.3: EMSAs of both variants of the -14028 T>C SNP in direct comparison. The phosphoimaging pictures of PAA gelshift assays show the binding of the labelled probes to proteins of the Caco-2 nuclear extract. Competition experiments (Comp.) with unlabelled variant oligonucleotides for a specific competitor for Cdx-2 (a) or HNF-4 α (b) indicate binding specificity of these TFs, which is shown further by supershift experiments with antibodies (Anti-) against Cdx-2 (a) and HNF-4 α (b). A non-specific antibody was used as negative control (b). Note that lanes were cut out of the gel pictures but lanes are from the same gels. Bands were classified as non-specific binding when they were only present on some of the gels and were reduced or removed with the use of less extract.

4.5.2 Transcription factor binding at -13779 G>C

As can be seen in Figure 4.4, both variants of the -13779 G>C SNP gave similar strong DNA-protein bands that could be competed with the unlabeled probe containing the ancestral or derived allele in a similar way.

Both variant probes show a slight competition with the Cdx-2 (Figure 4.4a) and only the ancestral allele showed a minor change in band intensity with the Oct-1 and HNF-1 α competitors. A complete displacement was in contrast seen with the oligonucleotide containing an HNF-4 α binding site and binding of this transcription factor could be confirmed with the supershift of the complex formed with the HNF-4 α antibody. The shift was slightly stronger for the derived variant 13779C probe on Figure 4.4b but this could be due to the general stronger probe on that gel.

Further competition experiments with Affymetrix competitor oligonucleotides for the predicted transcription factors LEF-1, GKLF-1 and AML-1 only revealed a slight competition with the GKLF-1 and AML-1 competitors similar for both, the ancestral and derived allele probes (not shown).

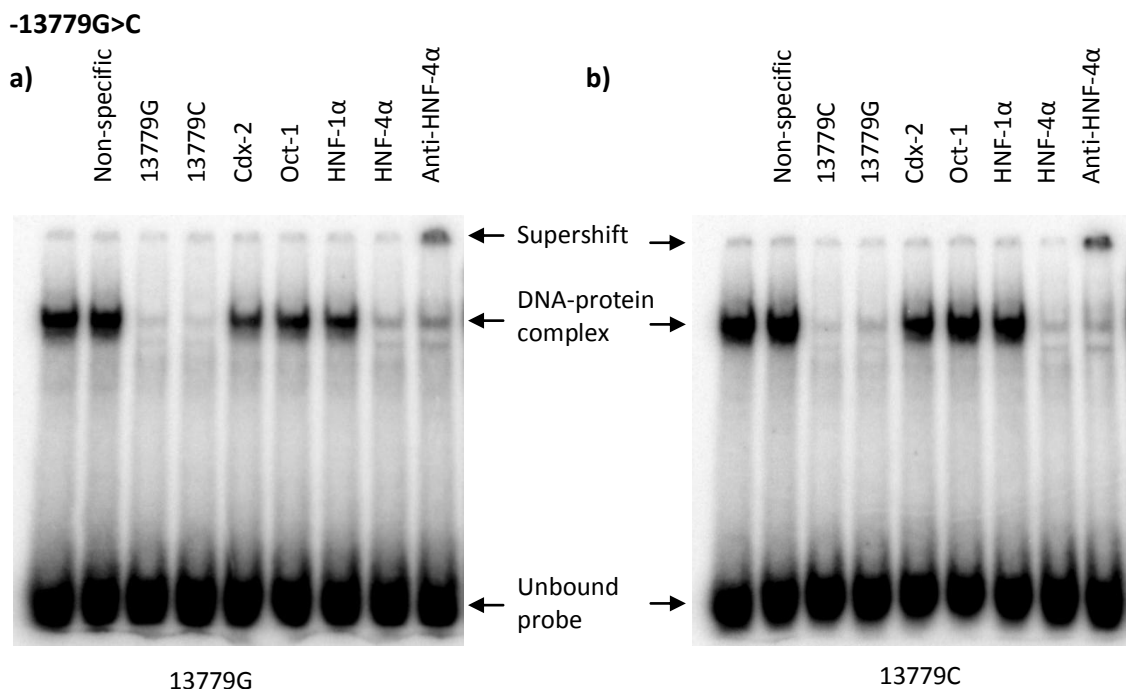


Figure 4.4: EMSA pictures of competition and supershift experiments for the ancestral (a) and derived (b) variant probes of the -13779G>C SNP. Competition experiments with unlabelled variant oligonucleotides, oligonucleotides with a non-specific sequence and binding sequences for different TFs are shown. These, as well as an antibody (Anti-) against the HNF-4 α TF for a supershift experiment, were used as indicated above the pictures. The specific DNA probe-protein complex could be clearly supershifted with this antibody (strong band on top of the gel).

4.5.3 Transcription factor binding at the positions -14011, -14010 and -14009

In a collaborative project, lead by Tine Jensen of the Copenhagen lab, we had identified transcription factor binding sites surrounding -14010G>C. The SNP is located in an Oct-1 binding site (-14021 to 14009) and resides close to an HNF-1 α binding site (-14007 to -13993). The lactase persistence associated -14010*C showed stronger binding to Oct-1 than the ancestral allele. However, this effect could only be shown with oligonucleotides spanning both transcription factors, indicating that it is mediated by HNF-1 α (Jensen et al. 2011).

Since -14011*T and -14009*G are located adjacent to that variant and are part of the same Oct-1 binding site, it seemed likely they would cause a similar effect. The oligonucleotides carrying the two alleles were therefore designed to cover the same length of sequence, including the Oct-1 and HNF-1 α binding sites as the 14010C probe (Jensen et al. 2011), and this probe was added to the assays for comparison.

Gel shift analysis indeed revealed similarities of all variant probes in forming protein complexes, visible as several bands on the gels. The upper band was competed in a similar way for the ancestral probe and the three variant probes with unlabelled Cdx-2, HNF-1 α and Oct-1 competitors (partly shown in Figure 4.5a). The strongest competition was visible with the HNF-1 α oligonucleotide, followed by the one containing an Oct-1 site and only minor competition was shown with the Cdx-2 competitor.

The 14011T probe showed the same strong protein binding as the 14010C probe, a slightly more intense band than the ancestral variant probe (14011C). Additionally, the supershift with an Oct-1 antibody, generated with all variant probes, was slightly stronger for the derived 14011T probe, similar to 14010C (Figure 4.5b). With the Cdx-2 and HNF-1 α antibodies a slight inhibition of the formation protein probe complex for all probes and in a similar way (not shown).

A slight difference seen for 14011T, compared to the other probes, was a slightly faster migrating DNA-protein complex visible as the lower band on the gels. Additional binding of GATA-3 and 4 to -14011*T was predicted bioinformatically and gelshift assays were performed to test this. Preliminary results show a competition of both 14011 probes with different GATA competitor oligonucleotides, which mainly affected the upper bands on the

gels (not shown). However this has not yet been confirmed with further experiments or in supershifts.

The 14009G probe showed a very interesting pattern that differed from the three other probes tested. In addition to a weak upper band, which could be shifted with the Oct-1 antibody and slightly inhibited with the Cdx2 and HNF-1 α antibodies, another DNA-protein complex was formed (shown by blue arrows in Figure 4.5). This suggests that besides the two other transcription factors binding to the 14009G probe, another protein in the nuclear extract is specifically binding to the -14009*G containing sequence..

-14011/10/09

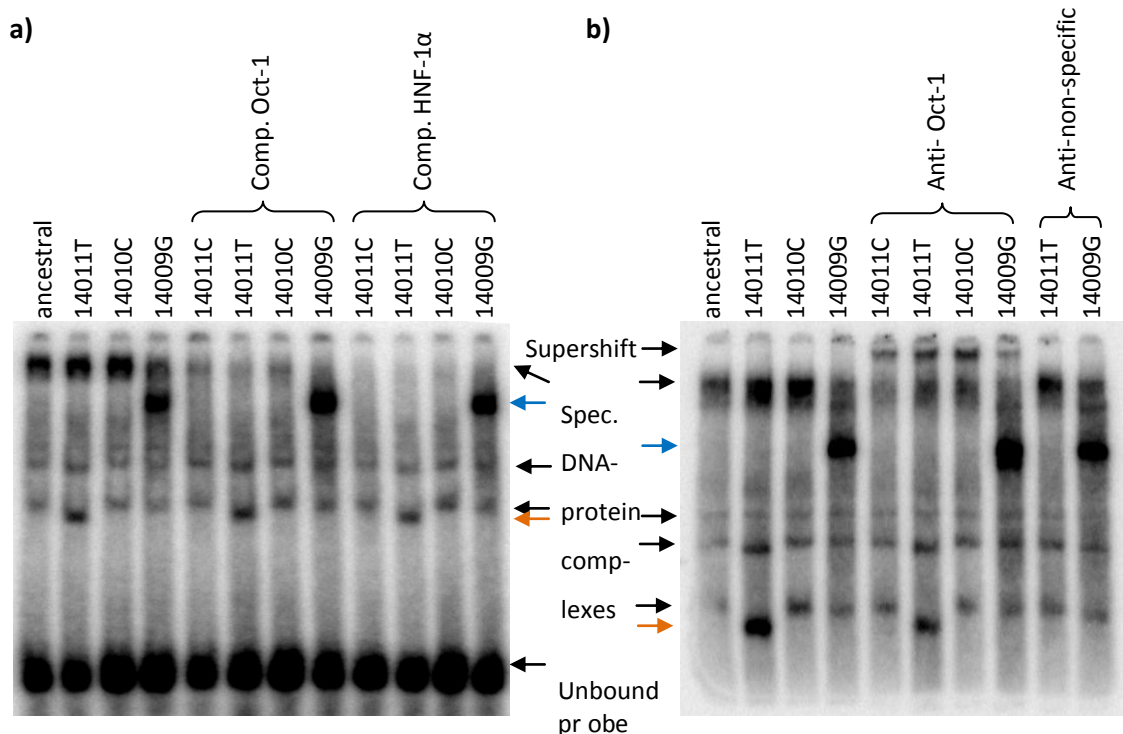


Figure 4.5: Phosphoimaging pictures of gelshift assays of competition (a) and supershift experiments (b) for the 14011T, 14010C and 14009G variant probes compared to the ancestral version. Competitors (Comp.) covering known binding sequences for TFs and antibodies (Anti-) were used as indicated above the gel images, a non-specific antibody was used as negative control. Different specific probe-protein complexes were formed of which the upper ones could be competed with Oct-1 and HNF-1 α competitors (a) and supershifted (b) with an Oct-1 antibody (red arrow) for all 4 probes, whereas the DNA-protein complex formed with the 14009G probe (blue arrow) was not shifted. A slightly different binding pattern was seen for the lower band for 14011T (orange arrow).

4.5.4 Binding of an Ets transcription factor to -14009*G

Bioinformatic analyses with different software programs (see section 2.3.13) predicted additional possible transcription factors that would bind to the 14009G oligonucleotide sequence, mainly members of the Ets transcription factor family. Further investigation of this sequence with the TRANSFAC database with a decreased matrix match threshold of 0.70 revealed further candidate transcription factors, as shown Appendix E, that potentially bind to the derived but not the ancestral variant probe for the 14009T>G SNP and are expressed in Caco-2 cells. Competitor oligonucleotides were designed taking into account specific transcription factor binding motifs, found in TRANSFAC (Table 4.1, above).

Table 4.1: Design of competitor oligonucleotides for EMSA experiments examining the -14009 T>G SNP. Information about candidate transcription factors, with high differences in MATCH scores between the ancestral and derived variant, were taken from the TRANSFAC database. Letters in capitals represent the core sequence of the binding matrix of a certain TF stored as identifier sequence in TRANSFAC.

Transcription factor	TRANSFAC consensus sequence	Matrix accession number	Identifier	Designed Oligonucleotide sequence	Name
c-Ets-1 (p54)	NNNRCCGGAWRYNNNN NNACMGGAWRTNN	M01078	V\$CETS1P54_03 V\$CETS1P54_02	aatcc TCTACCGGA tGTAGGTcgac	c-Ets-1
Ets	ACTTCCTS	M00971	V\$ETS_Q6	atcttact ACTTCCT Cgctgaccgt	Ets/Tel-2
NF-kappaB	NNNNKGGRAANTCCCN	M00774	V\$NFKB_Q6_01	agt GGCGGGGAA gTCCCCagaatc	NF-kappaB
Pax	CTGGAACMAC	M00808	V\$PAX_Q6	aagaag TGGAAC CACgatcgtgct	Pax
Pax-4	NGNVGTCANGCGTGNNNNYN	M00373	V\$PAX4_01	ac GGCGTTCATGCGTGAGCGACC gt	PAX4-8
Pbx	GATTGATKGNNNS	M00998	V\$PBX_Q3	agatgg GATTGATGGTAG ccgtatt	Pbx
Tel-2	YTA CT CCTG	M00678	V\$TEL2_Q6	see Ets	

Figure 4.6 shows the EMSA experiments with the first set of oligonucleotide competitors. It can clearly be seen that the upper DNA-protein complex could be competed with the ancestral sequence on both variant probes. The lower complex band with the -14009G probe could not be competed with the ancestral variant competitor, which confirms the specificity of the factor binding to the derived allele. The c-Ets-1 sequence was only one that competed strongly with the lower complex of 14009G. A slight competition of the binding of both variant probes to the upper band was seen for NF-kappaB, Pax, Pax4-8 and Pbx.

From the Affymetrix competitor set two oligonucleotides competed with the 14009G specific band: Aff_c-Ets-1 and Aff_ELK-1. However, the protein binding to the ancestral probe 14011C (shown as band for the specific DNA-protein complex in Figure 4.6a) was also competed with the Aff_ELK-1 competitor and slightly with Aff_ELF and Aff_ETS (1) competitors (not shown).

The reciprocal experiments, using c-Ets-1 and Aff_ELK-1 as probes showed that Aff-ELK-1 was competed with both competitor sequences, 14011C and 14009G, whereas the c-Ets-1 probe could only be completely competed with 14009G and only slightly with the ancestral version (not shown). So far it was not possible to confirm this in supershift experiments when an antibody for Ets1/2 or Ets-1 was added to the assay.

-14009 T>G

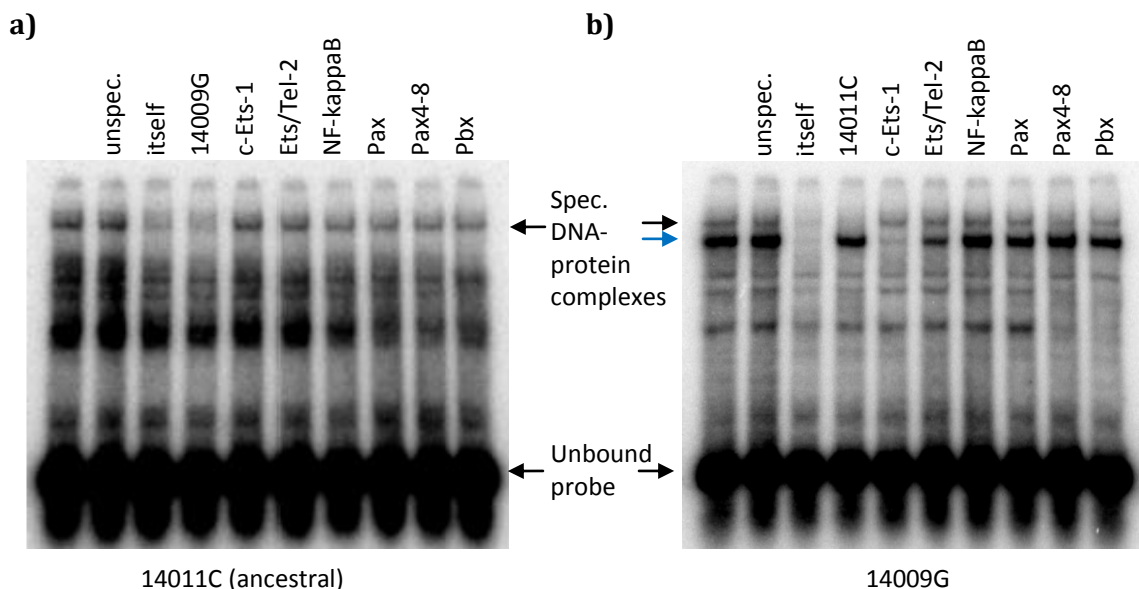


Figure 4.6: Images of gelshift assays of competition experiments for the ancestral (a) and derived (b) variant probes of the -14009 SNP. Competitors were used as indicated above the pictures. The specific DNA-protein complex for 14009G (blue arrow) could not be competed with the ancestral sequence competitor but was with competitor oligonucleotides of the Ets family.

4.6 Experimental strategy for reporter gene assays

Different strategies were pursued for the insertion of the *LCT* enhancer and the promoter variants into the reporter gene plasmids, upstream of the firefly luciferase gene (*luc+*), as described in chapter 2. Results for the experiments with the *LCT* promoter variants are discussed in chapter 6. Enhancer variants were generated via site directed mutagenesis and inserted via enzyme digest into the luciferase reporter vector pGL3 hLPH1085 (Troelsen et al. 2003) as shown in Figure 4.7, about 2.8 kb upstream of the human *LCT* promoter, already present in the construct. Transient transfections of Caco-2 cells were conducted as described in chapter 2 (section 2.2.7).

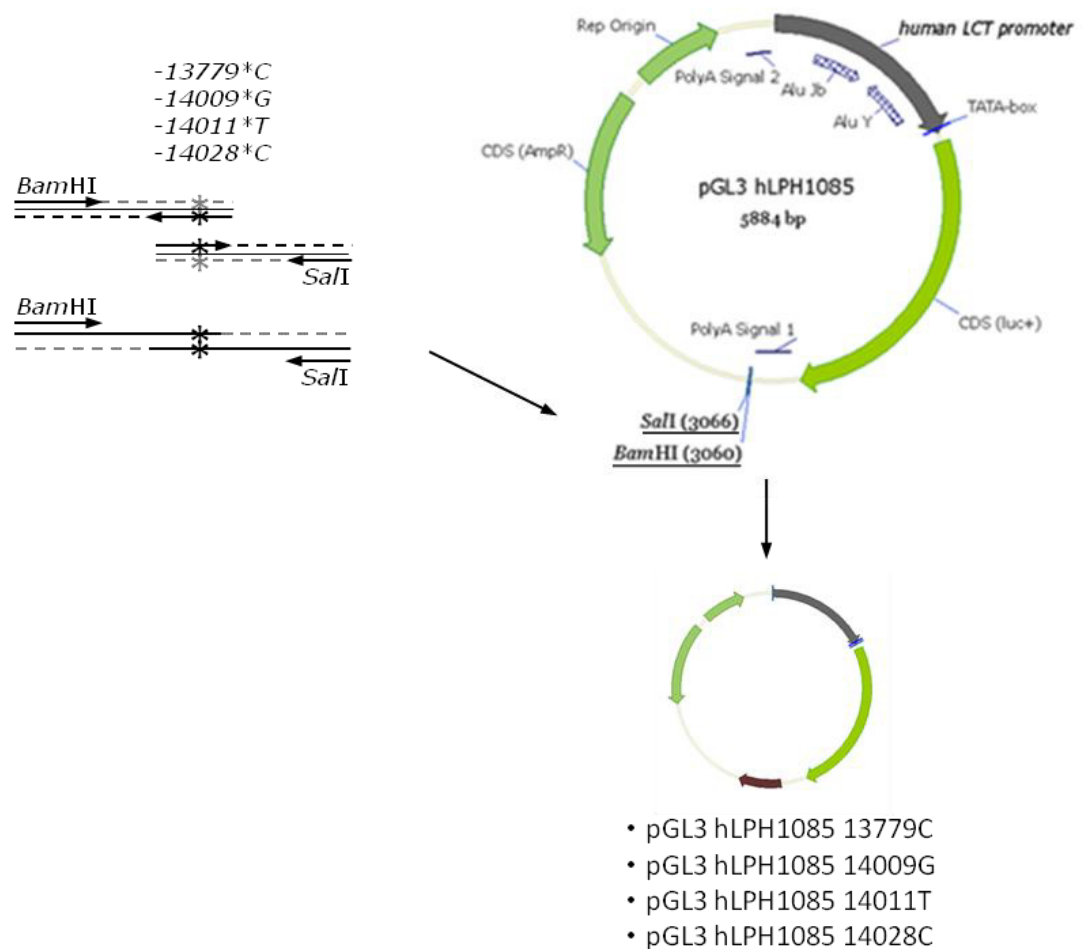


Figure 4.7: Summary of the site directed mutagenesis and insertion of *LCT* enhancer fragments into the luciferase reporter gene plasmids, as described in detail in section 2.2.7 (modification of Figure 2.2 of chapter 2). *LCT* enhancer variants were generated in a two step PCR amplification and inserted via *Bam*HI and *Sal*I into the pGL3vector containing the ancestral promoter region (pGL3 hLPH1085 (Troelsen et al. 2003)).

4.7 The influence of *LCT* enhancer variants on reporter gene expression

Luciferase reporter gene expression of the different enhancer variant constructs was measured in undifferentiated (2 days after transfection) and differentiated Caco-2 cells (9 days after transfection) and results of one experiment are shown in Figure 4.8. The -13910*T and -14010*C constructs were used as controls since they had previously been reported to influence reporter gene expression, at least in undifferentiated cells (Tishkoff et al. 2007; Troelsen et al. 2003).

The function of the 450 bp region carrying the ancestral alleles (Lewinsky et al. 2005; Troelsen et al. 2003) was confirmed since it enhanced *LCT* promoter function 7 fold in undifferentiated and 13 fold in differentiated cells compared to the promoter only constructs.

Significant differences in luciferase expression compared to the ancestral -13910*C constructs were demonstrated for all four newly tested variants in undifferentiated Caco-2 cells (Figure 4.8). The expression was about 1.3 times higher for the -13910*T, -14009*G and -14028*C reporter gene plasmids than the ancestral construct. For -14010*C and -14011*C this difference was even larger, up to about 1.4 fold expression compared to the ancestral version and the construct with -13779*C shows about twice the activity of the ancestral enhancer.

The picture changes slightly with the measurements in the differentiated cells, harvested 9 days after transfection. Only the activities for the control plasmids, -13910*T and -14010*C and those carrying -14009*G and -14011*T stayed significant (Figure 4.8). -13910*T increased luciferase activity to 1.8 fold compared to the ancestral enhancer and the effect of -14010*C was even bigger. It increases enhancer activity up to 2.8 fold in differentiated cells.

The effect of -14009*G on reporter gene expression resulted in a 2 fold increase in activity. The significant effects of -14009*G and -14010*C were also confirmed in another 9 day experiment, which was recently published (Jones et al. 2013).

The enhancer with the -14011*T variant is 1.4 (2 days) to 2 fold (9 days) more active than the ancestral version, which was also confirmed in another 9 day experiment (not shown).

These results support those of the gel shifts and suggest that both alleles (-14009 and 14011) alter lactase expression.

Although the constructs with -14028^*C and -13779^*C showed a 1.3 and 2 fold increased expression in the 2 day experiments, they did not show a significant increase in activity in the 9 day experiments. Both variants also revealed a high variation in luciferase activity in further experiments (data not shown) so it was not possible to confirm these results in the undifferentiated stage.

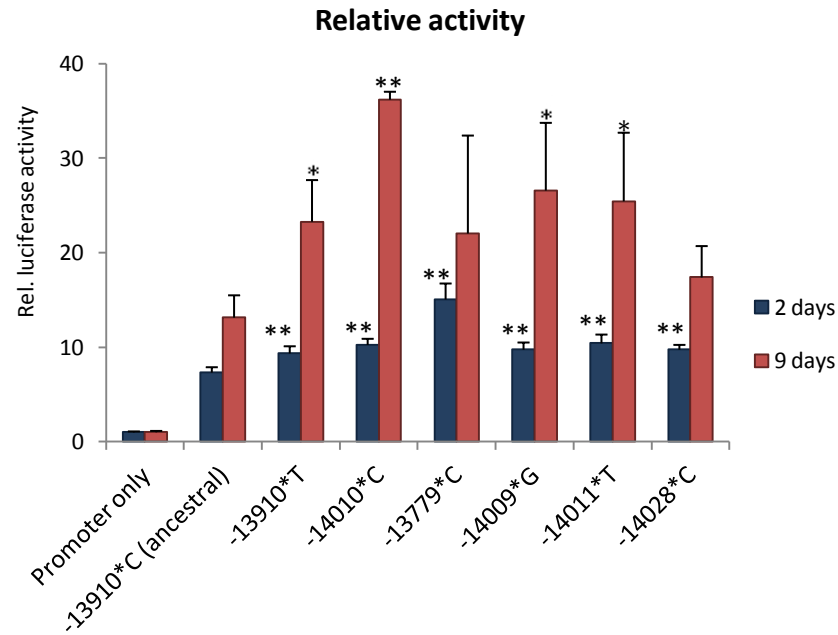


Figure 4.8: Result of luciferase reporter gene assays for *LCT* enhancer variants after 2 and 9 days of transfecting the Caco-2 cells. Luciferase activity of enhancer variant constructs are shown relative to the promoter only construct. Luciferase activities (means \pm SD, n=4) were corrected for transfection efficiency against β -galactosidase and normalised to the expression of pGL3 hLPH1085 (promoter only). Significant differences of luciferase activity compared to the ancestral variant were calculated using a student *t* test and are shown by ** ($p < 0.01$) and * ($p < 0.05$).

4.8 Summary and discussion of the functional studies

Functional evidence for the 4 variants -14028*C, -14011*T, -14009*G and -13779*C in comparison to their ancestral alleles, obtained from EMSA and transfection experiments that were conducted during this thesis work are summarised in Table 4.2. A more detailed summary of the experimental outcome and bioinformatic investigations for each of these variants can be found in Appendix E.

Table 4.2: Summary of the main outcome of the functional studies conducted during this thesis. Abbreviations: +: variant has an effect on reporter gene expression or shows binding to a certain transcription factor, bold crosses: strong effect, - no effect, (+) weak effect respectively, n/a not applicable.

	-14028*T	-14028*C	-14011*C	-14011*T	-14009*G	-13779*G	-13779*C
Transfections							
2 days	control	+	control	+	+	control	+
9 days	control	-	control	+	+	control	-
EMSAs							
Cdx-2	+	+	+	+	+	(+)	(+)
Oct-1	+	+	+	+	+	(+)	-
HNF-1 α	+	+	+	+	+	(+)	-
HNF-4 α	-	+				+	+
c-Ets-1	n/a	n/a	-	n/a	+	n/a	n/a
ELK-1, ETS	n/a	n/a	+	n/a	+	n/a	n/a

It was clearly shown that sequences containing the two alleles, -14011*T and -14009*G differ functionally from their ancestral forms *in vitro*, as concluded from transfection experiments and evidence from EMSAs. The high activity of the enhancer constructs carrying those alleles, compared to the ancestral variant, could be explained by an altered binding to different transcription factors in each case: Slightly stronger binding of -14011*T to Oct-1 and probably also stronger binding to a GATA factor and the binding of -14009*G to an additional transcription factor, most probably a member of the Ets family.

Binding of -14009*G to the Ets-1 transcription factor could not be confirmed in EMSA experiments with either of the Ets-1/2 and Ets-1 antibodies. However, these commercially available antibodies might not be specific enough or even suitable for EMSA experiments and at the time no other Ets antibody was available that did have confirmed function in binding to Ets-1 in EMSA studies.

Although direct evidence from supershift assays is missing the competition with commercially available and target designed competitors strongly suggest the binding to c-Ets-1 or a member of the same transcription factor family. Ets (E26 transformation-

specific) factors play an important role in the differentiation, survival and proliferation of cells. These factors usually interact with other factors to regulate DNA-binding or transcription (for example Sharrocks 2001) and could well play a role in the regulation of *LCT* expression.

The -14028 variant tested apparently slightly influenced *LCT* promoter activity in luciferase reporter gene assays (but only significantly in undifferentiated Caco2 cells) and the results were not replicated during the course of this work. There was however some evidence for alteration of transcription factor binding in EMSAs. It could be shown that the derived -14028*C leads to a loss of binding to Cdx-2 and seems to create a HNF-4 α binding site, but it can be concluded that this does not seem to change enhancer activity dramatically *in vitro*, at least under the conditions tested.

In contrast to all other variants tested, a direct functional effect was not supported for -13779 G>C. The probes were shown to contain an HNF-4 α binding site, which is probably the cause for the footprint around that position shown in Figure 4.2, but the -13779 G>C polymorphism doesn't seem to have an effect on protein binding. Mutation analysis might be useful to define the exact binding site of this transcription factor along sequence the studied here, but there is no real hint that this particular substitution has any effect. The suggestive evidence for a functional role of -13779*C had been not very strong as it mainly came from its relatively high frequencies in milk drinking populations in India, but it had in fact previously been reported in one Somali individual who was diagnosed as a lactose maldigester (Ingram et al. 2009b).

The results show that -14011*T and -14009*G, at least, give clear evidence of a difference *in vitro* but it should always be kept in mind that this does not necessarily prove a functional difference *in vivo*. Physiological conditions are different in the living organism and other factors influence DNA-protein binding, such as chromatin structure or other flanking sequences of importance that cannot be mimicked in EMSA experiments or transfection studies. It might also be that *in vivo* function of the variants is even larger as in the transfection experiments as Caco2 is only capable of expressing small amounts of lactase in comparison to the cells of the small intestine (Rousset 1986). In the case of -14009*G, the association with lactase persistence further confirms its likely functional role. Unfortunately -14011*T was not frequent enough in any one population to make this approach a possibility.

Techniques based on chromatin-immunoprecipitation such as ChIP-chip (chromatin immunoprecipitation coupled to microarray hybridisation) or ChIP-Seq (chromatin immunoprecipitation coupled to high throughput sequencing) give a better insight to specific transcription factor DNA targets and their networks in the intestine (reviewed Olsen et al. 2012) and could be useful to further examine the influence of the *LCT* enhancer alleles on transcription factor binding.

Further experiments would also be necessary to investigate the interaction of the identified transcription factors with other factors involved in lactase expression. Previous studies revealed interesting results. For example was luciferase activity highest for constructs containing -13915*G under simultaneous over-expression of Oct-1 and HNF-1 α but less with HNF-1 α and Oct-1 alone (Enattah et al. 2008). A similar interaction of these two transcription factors in mediating *LCT* enhancer activity was previously shown for constructs containing the -13910C>T polymorphism (Lewinsky et al. 2005).

The transcription factors predicted bioinformatically only overlap to a certain extent with those successful confirmed experimentally. Bioinformatic tools are useful in searching for candidate transcription factors for functional tests, nevertheless they have their limitations as most of the binding matrixes are collected from different organisms and cell types. However, data about transcription factor binding sites will increase dramatically in the next years with the application of new techniques and large scale investigations of functional elements in a great range of cell types in project such as ENCODE (Hoffmann et al. 2012), which will make predictions more accurate.

During the course of this project the question was posed as to whether there were any other polymorphic changes (reflecting the different haplotype background of the variants) that might influence the level of expression in our *in vitro* experiments. To check this the sequence of the promoter used as part of the construct was reviewed in detail and was shown to derive from a B haplotype chromosome. It should thus be noted that only -14028*C is tested in a construct with a promoter sequence reflecting its *in vivo* situation, with a T* allele at position 958, which is part of the B haplotype background of the variant. All other reporter-gene-constructs that were used to test the effect of certain enhancer variants were done using the same pGL3 hLPH1085 vector (Troelsen et al. 2003). Since it had been reported previously that this SNP alters transcription factor binding in gel shift assays (Hollox et al. 1999) this site was inspected bioinformatically and shown to be located within a predicted Oct-1 site. Experiments were therefore conducted to attempt to

replicate the gel shifts. These showed a gel shift which was stronger for the ancestral than the derived variant and could be slightly competed with Oct-1 competitor oligonucleotides. However, these results are only preliminary and would need further confirmation.

A clear line for future research would be to analyse the possible combinatorial effect of this allele with the various enhancer alleles.

Lactase is only fully expressed in differentiated cells and therefore the activity measurements of the cells harvested 9 days after transfection might better represent the influence of the variants. The differences in expression from 2 to 9 days for all enhancer variants tested show that the mean luciferase activity rises more than 2 fold from the undifferentiated to the differentiated cell state for these variants showing a significantly different expression to the ancestral variant of the enhancer. One can speculate that it is the ability of a variant to up-regulate expression during the differentiation process that is the crucial effect for lactase persistence. Unfortunately, it is not possible to study the maturation of cells and the influence of the lactase persistence associated alleles on expression during that development *in vitro*. Model organisms could be used to study this as recently done for -13910*T in mice (Fang et al. 2012). However for cost and logistic reasons the only practical ways to study several novel alleles is the combination of *in vitro* functional studies, as was done here. For any that are more frequent, phenotype association studies, perhaps in combination with mRNA expression, is highly informative but cannot provide direct evidence because of linkage disequilibrium

5 Evolutionary background of LCT enhancer variants

5.1 Introduction

In chapter 3, a number of different derived enhancer variants were documented, in a large number of European and Asian populations. This chapter aims to combine information from several sources, about the common enhancer variants and determine their haplotype background to get a better picture about their evolution and possible signatures of selection in the genetic regions examined.

Previous studies by different members of our group, which attempted to determine the core *LCT* haplotype background of the different *LCT* enhancer variants used slightly different combinations of genetic markers, and more limited sample sets (Ingram et al. 2009b; Jones et al. 2013; Poulter et al. 2003). The next step in getting a better and more consistent picture about the haplotype background of enhancer variants would be to add more and missing markers and to combine more information about populations from Africa, Europe, the Middle East and other parts of Asia in the analysis.

It is widely agreed that the lactase persistence associated alleles occur on more than one haplotype, which was part of the evidence that lactase persistence evolved independently several times (Enattah et al. 2008; Ingram 2008; Ingram et al. 2007; Tishkoff et al. 2007), but it is less clear whether the same mutations might have recurred. One publication claimed a different haplotype background for some of the *-13910*T* alleles in groups of Russia and Iran and therefore concluded that this site had mutated more than once (Enattah et al. 2007). It is of interest to see if there is any evidence for that from looking at similar extended haplotypes in a different dataset, but including Iranians, which might or might not support this suggestion.

The B haplotype, which is quite common across the globe (Hollox et al. 2001), showed evidence of a frequent extended haplotype across at least 500 kb in a collaborative study focussing on South Asia (Gallego Romero et al. 2012), see Appendix G. My contribution to this work involved extracting SNP data from families of the 'Human Genome Diversity Panel-Centre d'Etude du Polymorphisme Humain ' (CEPH) (Dausset et al. 1990) from

previous work in our lab and merging it with public data for the CEPH (CEU) samples from the HapMap project (<http://hapmap.ncbi.nlm.nih.gov/>) in order to infer which were the B haplotypes.

We were curious that this haplotype was so frequent yet and did not carry any of the main derived lactase persistence associated alleles. Only the *-14028*C* variant on a high lactase expressing chromosome was associated with the core B haplotype (Poulter et al. 2003), but as seen in chapter 3 it is not frequent in any of the populations studied. This led to suggestions that the length of chromosome known as the B haplotype might have been selected for by another advantageous mutation located on this sequence.

5.2 Chapter aims

Thus this chapter aims to examine the extended haplotype background of the derived enhancer variants in this combined dataset from various geographic regions, with the target of obtaining further insight into the demographic history and origin of these alleles. I also investigate the distribution of the extended B haplotype to get insights into the selective or demographic explanations for its frequent appearance. I examine patterns of linkage disequilibrium and also test methods to detect evidence for different types of selection.

5.3 Population selection

Of the new dataset described in chapter 3, the Middle Eastern populations showed the most variation across the *LCT* enhancer region. Middle Eastern sample sets (Bedouin groups, Israel and Palestinian Arabs) previously analysed by our group (Ingram et al. 2007) were also included for further analysis as well as randomly chosen samples from several African populations (Afar, Beni Amer, Chagga) that showed high frequencies of lactase persistence associated alleles (Jones 2012). Of interest were also populations (Asante, Chewa, Mambila, Shabo) that do not have a pastoralist lifestyle (see also chapter 2, Table 2.2), in which enhancer variation was mainly absent (Jones 2012).

To get a wider picture of the haplotype distribution, several European and Asian groups, analysed in chapter 3 were included in the sample set.

To look specifically at differences between lactase persistent and non-persistent individuals and compare with previous findings, phenotyped samples for Europe (Northern Europeans and Italians) and Africa (Amhara, Oromo, Jaali) were also part of the dataset.

5.4 Sequencing/Genotyping strategy

Five regions in and around the *LCT* gene were included in the initial haplotype analysis spanning a region of about 80 kb, as shown in Figure 5.1.

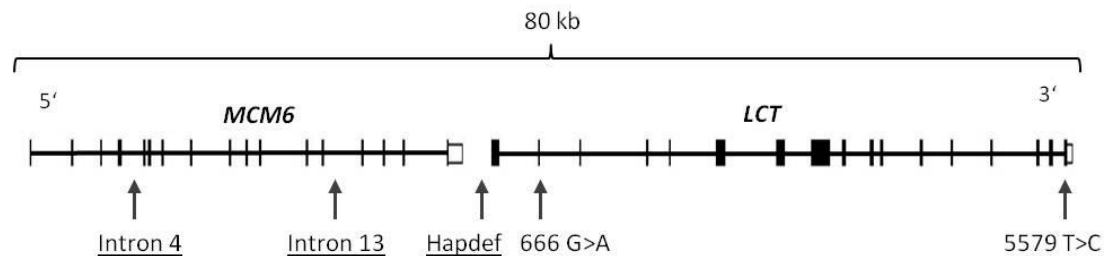


Figure 5.1: Sequence regions and SNPs included in haplotype analysis, spanning a region of 80kb.

To obtain as much as possible of the *LCT* core haplotype information as described by Hollox (2001) the haplotype defining region upstream of *LCT*, called the 'hapdef' region within this thesis as it was in Jones et al. (2013), was sequenced and information about the two haplotype markers in exon 2 and exon 17 of *LCT*, 666 G>A and 5579 T>C mainly obtained via genotyping was also collected as described below. The *MCM6* intron 4 region also described in Jones et al. (2013) was also sequenced to get haplotype information in relation to intron 13 of *MCM6*. The hapdef and intron 4 information was also used to serve as control regions for the enhancer to compare for diversity. Both regions are located near enough to the enhancer to share a similar history, with low recombination between them and little difference in mutation rates (Jones et al. 2013).

All sequencing data used in this chapter that formed part of previous projects of the group were re-analysed and sequencing repeated to get complete datasets, for example to extend the *LCT* enhancer region further 3', or if readable sequences were not found. All Middle Eastern samples analysed by Kate Ingram (Ingram et al. 2007) and those of chapter 3, the European and Asian groups with complete enhancer data were sequenced for the *MCM6* intron 4 and *LCT* hapdef regions specifically for this project. Additional samples of the Saudi Bedouins were included as well and sequenced for all regions.

The hapdef sequence region upstream of the *LCT* promoter spans 701 bp (Chr2:136595212-136595912, GRCh37/hg19). This region was successfully amplified with primers applied previously (Jones et al. 2013) and sequences were readable for all samples from bp position -1100 until -630 from the start of transcription of *LCT*. An InDel

at the 3' end of the sequence and the consequent need for a different internal sequencing primer restricted the length of evaluable sequence region. The control region of *MCM6* intron 4 covers a 683 bp region (chr2:136624515-136625197, GRCh37/hg19) and sequences were readable from bp position -30292 to -29922 upstream of the *LCT* transcription start. The sequences for the *LCT* enhancer region were obtained as described in chapter 3.

The haplotype markers 666 G>A and 5579 T>C were part of the marker set (see below), genotyped by LGC Genomics (KBioscience). The regions of exon 2 and 17 of *LCT* were also subjected to sequencing for a subset of the Middle Eastern samples within an undergraduate project of Lana Couzens (Couzens 2011). These data were used to crosscheck for quality of the genotype data delivered by LGC Genomics and to complement missing data.

Details about all primer sequences can be found in Table 2.3 of chapter 2.

5.4.1 Marker selection for extended haplotype analysis

Markers for extended haplotype analysis were chosen to span a sequence region of about 1.8 Mb around *LCT*. Certain criteria were deployed to get an informative marker selection. All SNPs are part of the Illumina 650k platform typed for most of the HapMap populations but also used for the haplotype analysis in our recently published paper about Indian and European cattle herders (Gallego Romero et al. 2012). With the haplotype information of these data it was possible to exclude close SNPs in complete LD with each other. To further guide the choice of markers LDU maps were constructed (see below, 5.5.8.3), that were particularly useful in the region downstream of *LCT* where there is a long region of very high LD between the genes *R3HDM1* and *RAB3GAP1*.

The first set of markers was chosen to be broadly 50 kb apart, with the distance increasing for the outer SNPs, to have a minor allele frequency of at least about 8% and to be as different as possible in frequency between the CEPH (European), Yoruban and Han-Chinese HapMap populations. Downstream of *LCT* the region was extended up to about position 135.8 Mb (b37, hg 19) on chromosome 2 to include the region where a sharp drop in extended haplotype homozygosity (EHH) was seen in European and Asian populations (Gallego Romero et al. 2012). Later the *LCT* haplotype SNPs 666 G>A (rs3754689) and 5579 C>T (rs2278544) were also included, as well as the lactase persistence associated SNP 22kb upstream of *LCT* (-22018 G>A, rs182549).

The chromosomal location and further information about the 36 SNPs chosen can be found in Figure 5.2 and Table 5.1.

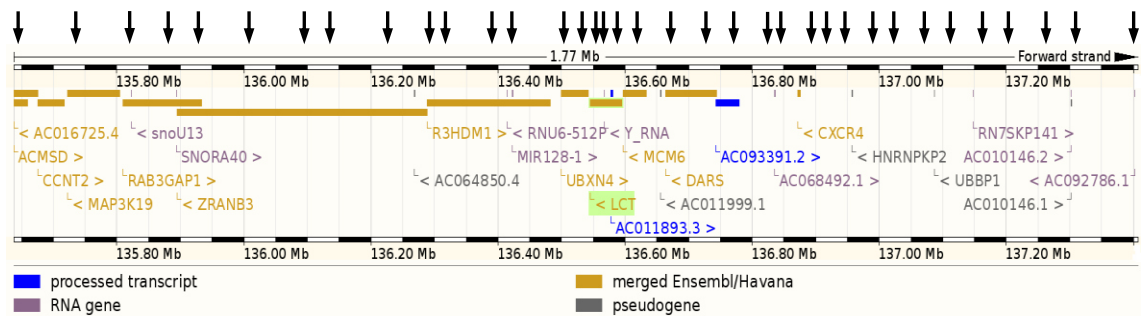


Figure 5.2: Location of the SNPs chosen for extended haplotype analysis. The broad position on chromosome 2 of all SNPs is indicated by arrows. The picture shows all genes documented on Ensembl for the chosen region and their position in chromosomal+ direction (b37), taken and modified from the Ensembl website (<http://www.ensembl.org>). Note that *LCT* and *MCM6* are transcribed from the chromosomal negative strand accounting for the location of *LCT* (highlighted in green) on the left of *MCM6*.

Table 5.1: Chromosomal location and allelic information about all 36 SNPs chosen for extended haplotype analysis.

		Position on Chromosome		Dist.	SNP	Anc.	MAF	MAF in HapMap pop.		
No.	SNP	b36 (Kb)	b37 (kb)	(kb)	(Chr +)	allele	1000 genomes	CEU	YRI	CHB
1	rs1446525	135354.317	135637.847		A/G	A	G = 0.288/629	0.60	0.16	0.07
2	rs4954209	135454.378	135737.908	100	G/T	T	T = 0.348/761	0.18	0.37	0.62
3	rs2874739	135535.377	135818.907	81	C/T	T	T = 0.347/757	0.12	0.75	0.20
4	rs1869829	135594.032	135877.562	59	A/G	G	A = 0.385/840	0.81	0.02	0.38
5	rs2305248	135644.782	135928.312	51	A/G	G	G = 0.343/748	0.12	0.74	0.20
6	rs1900741	135718.970	136002.500	74	C/T	T	C = 0.431/941	0.91	0.25	0.14
7	rs1561277	135808.531	136092.061	90	A/C	A	C = 0.264/577	0.74	1.00	1.00
8	rs9798267	135846.261	136129.791	38	A/G	A	G = 0.277/604	0.08	0.66	0.15
9	rs6709132	135949.042	136232.572	103	A/G	G	G = 0.211/460	0.08	0.31	0.15
10	rs3806502	136004.743	136288.273	56	C/T	T	T = 0.327/714	0.12	0.76	0.15
11	rs4954265	136040.695	136324.225	36	A/G	G	G = 0.270/590	0.06	0.67	0.15
12	rs961360	136110.128	136393.658	69	A/G	A	G = 0.315/687	0.08	0.30	0.43
13	rs4954278	136124.761	136408.291	15	C/T	C	T = 0.181/396	0.08	0.38	0.15
14	rs6430585	136223.397	136506.927	99	A/C	C	A = 0.292/637	0.14	0.30	0.22
15	rs10188066	136255.983	136539.513	33	A/G	G	G = 0.455/993	0.17	0.79	0.34
16	rs2278544	136262.580	136546.110	7	A/G	A	G = 0.492/1074	0.82	0.21	0.40
17	rs2304370	136278.205	136561.735	16	A/G	G	A = 0.254/555	0.11	0.38	0.20
18	rs3754689	136307.216	136590.746	29	C/T	C	T = 0.339/740	0.09	0.49	0.41
19	rs182549	136333.224	136616.754	26	C/T	C	T = 0.234/510	-	-	-
20	rs309152	136373.722	136657.252	40	A/G	G	G = 0.321/702	0.08	0.44	0.42
21	rs6430594	136435.643	136719.173	62	A/G	A	G = 0.198/432	0.09	0.09	0.16
22	rs309137	136482.421	136765.951	47	C/T	C	T = 0.376/821	0.79	0.02	0.35
23	rs2090660	136535.189	136818.719	53	C/T	C	T = 0.269/588	0.20	0.11	0.23
24	rs6430600	136552.835	136836.365	18	A/G	A	A = 0.343/749	0.29	0.62	0.19
25	rs12691874	136596.944	136880.474	44	A/G	G	A = 0.339/740	0.60	0.10	0.19
26	rs953387	136623.640	136907.170	27	A/C	A	T = 0.460/1004	0.71	0.56	0.16
27	rs1016269	136657.132	136940.662	33	A/G	G	A = 0.279/610	0.17	0.24	0.38
28	rs7371043	136693.406	136976.936	36	C/T	T	T = 0.158/344	0.08	0.18	0.30
29	rs4074120	136743.057	137026.587	50	C/T	T	C = 0.371/810	0.20	0.28	0.51
30	rs12465599	136791.320	137074.850	48	A/G	G	G = 0.439/958	0.61	0.55	0.27
31	rs6715450	136838.201	137121.731	47	A/G	A	A = 0.346/756	0.27	0.35	0.40
32	rs543721	136878.027	137161.557	40	G/T	G	T = 0.411/897	0.37	0.26	0.58
33	rs12618749	136921.944	137205.474	44	C/T	C	T = 0.228/497	0.09	0.20	0.41
34	rs16834591	136979.740	137263.270	58	A/G	A	A = 0.260/567	0.09	0.31	0.37
35	rs580879	137030.609	137314.139	51	C/T	T	T = 0.257/562	0.19	0.53	0.14

36	rs6711718	137123.482	137407.012	93	C/T	T	C = 0.451/985	0.50	0.48	0.20
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5.5 Results

5.5.1 Distribution of the variation of *LCT* enhancer and flanking regions in a combined population set

Full data for the three sequenced regions (intron 4 and intron 13 of *MCM6* and the haplotype defining region hapdef of the *LCT* promoter) and the additional two haplotype markers 666 G>A and 5579 T>C were obtained from a final dataset of 872 samples. This sample set also contained 7 single individuals from different sample groups with rare enhancer SNPs, which were included in later haplotype analyses. 865 samples belonging to 28 populations were analysed with the Arlequin software and all variants were checked, in each of the 28 populations, for deviation from HWE, mainly to reassure the exclusion of sampling bias and genotyping problems. Only in four populations was a borderline *p*-value observed for single SNPs, which did not remain significant after Bonferroni correction for 28 tests.

Table 5.2 to Table 5.4 summarise the allele frequencies for the SNPs within the three sequenced regions and the two haplotype SNPs for the 28 populations.

Table 5.2: Allele frequencies for the SNPs in the *MCM6* intron 4 region in the combined sample set of 28 populations, N: Number of chromosomes.

Region	Country	Populations	N	rs1435577 -30210 G>C	- -30203 G>Del	- -30196 A>G	rs56263017 -30182 A>G	- -30160 A>T	- -30071/70 TC>AA	rs4988172 -29949 G>C
Africa	Cameroon	Mambila	40	0.950	-	-	0.200	-	-	0.075
		Ethiopia	124	0.677	-	-	0.242	-	-	-
		Amhara	80	0.588	0.025	-	0.250	-	-	-
		Oromo	124	0.500	-	-	0.210	-	-	0.024
		Shabo	44	0.841	-	0.023	0.318	0.091	-	0.159
	Ghana	Asante	40	0.825	-	-	0.200	-	-	0.175
	Malawi	Chewa	40	0.875	-	-	0.350	-	0.025	0.150
	Sudan	Beni Amer	130	0.800	-	-	0.346	-	0.008	0.008
		Jaali	76	0.750	-	-	0.329	-	-	0.026
	Tanzania	Chagga	82	0.646	-	-	0.159	-	-	0.110
Central Asia	Mongolia	Khalka	38	0.789	-	-	0.132	-	-	0.053
	Nepal	Tharu	40	0.750	-	-	0.125	0.025	-	0.050
Europe	Northern Europeans		40	0.900	-	-	0.125	-	-	0.050
	Italy	Italians	32	0.656	-	-	0.219	0.063	-	0.063
	Norway	Norwegians	40	0.975	-	-	0.100	-	-	-
	Romania	Romanians	40	0.675	-	-	0.275	0.025	-	0.025
	Ukraine	Ukrainians	40	0.675	-	-	0.075	-	-	0.125
Middle East	Iran	Iranians	78	0.579	-	-	0.355	0.026	-	0.026
		Israeli Arabs	40	0.575	-	-	0.200	0.075	0.025	0.050
	Israel	Israeli Bedouin	32	0.719	-	-	0.375	-	-	0.063
		Palestinians	38	0.632	-	0.026	0.316	-	0.026	0.105
	Jordan	Jordanian Bedouin	44	0.682	-	-	0.409	-	-	-
	Kuwait	Kuwaiti	62	0.714	-	-	0.519	-	-	0.018
	Saudi Arabia	Saudi Bedouin	40	0.725	-	-	0.500	-	-	-
	Syria	Syrians	82	0.512	-	-	0.232	-	-	0.037
	Turkey	Anatolian-Turks	40	0.500	-	-	0.200	0.050	-	0.125
	Yemen	Yemeni Hadramaut	156	0.756	0.013	-	0.467	0.006	-	0.026
		Yemeni Sena	68	0.853	-	-	0.382	0.074	-	0.060

Table 5.3: Allele frequencies for the SNPs in the *LCT* 'hapdef' region and two haplotype markers in the combined sample set of 28 populations, N: Number of chromosomes.

Region	Country	Populations	N	rs56064699	rs148142676		rs78205226			rs56211644		rs3754689	rs2278544
				-958 C>T	-943 C>G	-943/42 TC>Del	-931 T>C	-875 G>A	-815 A>G	-811 A>G	-678 A>G	666 G>A	5579 T>C
Africa	Cameroon	Mambila	40	-	-	0.525	-	-	-	-	0.200	0.575	0.325
		Ethiopia	124	0.290	-	0.040	-	-	-	-	0.242	0.363	0.351
		Amhara	80	0.388	-	0.063	-	-	-	-	0.238	0.500	0.171
		Oromo	124	0.452	-	0.113	-	0.008	0.008	-	0.205	0.607	0.237
		Shabo	44	0.024	-	0.167	-	-	-	-	0.333	0.295	0.432
		Ghana	40	0.100	-	0.350	-	-	-	-	0.200	0.500	0.325
		Malawi	40	0.025	-	0.300	-	-	-	-	0.375	0.425	0.300
		Sudan	130	0.138	-	0.146	-	-	-	0.015	0.362	0.346	0.349
			76	0.211	-	0.132	-	0.026	-	-	0.329	0.382	0.276
	Tanzania	Chagga	82	0.098	-	0.341	-	-	-	-	0.159	0.695	0.200
Central Asia	Mongolia	Khalka	38	0.211	-	0.211	-	-	-	-	0.132	0.375	0.500
	Nepal	Tharu	40	0.289	0.026	0.289	-	-	-	-	0.118	0.600	0.300
Europe	Northern Europeans		40	0.100	-	-	-	-	-	-	0.100	0.111	0.789
	Italy	Italians	32	0.313	-	-	-	0.063	-	-	0.250	0.333	0.467
	Norway	Norwegians	40	0.025	-	-	-	-	-	-	0.100	0.025	0.875
	Romania	Romanians	40	0.325	-	-	-	0.100	-	-	0.225	0.325	0.500
	Ukraine	Ukrainians	40	0.325	-	-	-	0.025	-	-	0.075	0.316	0.600
Middle East	Iran	Iranians	78	0.410	-	-	-	0.013	-	-	0.308	0.410	0.333
		Israeli Arabs	40	0.400	-	0.025	-	-	-	-	0.200	0.450	0.400
		Israeli Bedouin	32	0.250	-	0.031	-	-	-	-	0.375	0.313	0.313
		Palestinians	38	0.342	-	0.053	-	0.026	-	-	0.237	0.421	0.306
		Jordan	44	0.318	-	-	-	0.068	-	-	0.409	0.341	0.250
	Kuwait	Kuwaiti	62	0.293	-	0.067	-	0.019	-	-	0.481	0.397	0.172
	Saudi Arabia	Saudi Bedouin	40	0.250	-	0.050	-	-	-	-	0.475	0.316	0.200
	Syria	Syrians	82	0.488	0.012	-	-	0.037	-	-	0.220	0.488	0.408
	Turkey	Anatolian-Turks	40	0.450	-	-	-	0.025	-	-	0.225	0.450	0.425
	Yemen	Yemeni Hadramaut	156	0.237	-	0.064	0.013	0.038	-	-	0.436	0.314	0.264
		Yemeni Sena	68	0.152	-	0.015	-	-	-	-	0.333	0.152	0.438

Table 5.4: Allele frequencies for the SNPs in the *LCT* enhancer region in the combined sample set of 28 populations, N: Number of chromosomes.

Populations	N	-	rs4988233	rs145946881	ss820486563	ss820496524	rs41380347	rs41456145	rs4988235	rs41525747	ss820496565	rs144412793	-	-	rs4954492	rs56348046	rs4954490
		-14028 T>C	-14011 C>T	-14010 G>C	-14009 T>G	-13957 A>G	-13915 T>G	-13913 T>C	-13910 C>T	-13907 C>G	-13806 A>G	-13800 G>T	-13779 G>C	-13744 C>G	-13730 T>G	-13603 C>T	-13495 C>T
Mambila	40	-	-	-	-	-	-	-	-	-	-	-	-	-	0.075	0.050	0.125
Afar	124	-	-	-	0.008	0.008	0.185	0.008	-	0.274	0.032	-	-	-	0.040	-	0.360
Amhara	80	-	-	-	0.038	-	0.050	0.013	-	0.025	0.025	-	-	-	0.050	-	0.241
Oromo	124	-	-	0.008	0.024	-	0.145	0.016	-	0.048	0.032	-	-	-	0.024	0.025	0.162
Shabo	44	-	-	-	-	0.023	-	-	-	-	-	0.023	-	-	0.023	-	0.114
Asante	40	-	-	-	-	-	-	-	-	-	-	-	-	-	0.075	-	0.075
Chewa	40	-	-	-	-	-	-	-	-	-	-	-	-	-	0.025	0.025	0.125
Beni Amer	130	-	-	-	0.138	-	0.262	-	-	0.169	-	-	-	-	0.047	-	0.230
Jaali	76	-	-	-	0.092	-	0.197	0.013	0.013	0.013	-	-	-	-	0.079	0.039	0.172
Chagga	82	-	-	0.146	-	-	-	-	-	-	-	0.012	-	-	0.061	-	0.063
Khalka	38	-	0.026	-	-	-	-	-	0.026	-	-	-	-	-	-	-	0.421
Tharu	40	-	-	-	-	-	-	-	0.075	-	-	-	-	-	-	-	0.211
Northern Europeans	40	0.025	-	-	-	-	-	-	0.650	-	-	-	-	-	-	-	0.737
Italians	32	-	0.031	-	-	-	-	-	0.031	-	-	-	-	-	-	-	0.344
Norwegians	40	-	-	-	-	-	-	-	0.850	-	-	-	-	-	-	-	0.875
Romanians	40	-	-	-	-	-	-	-	0.150	-	-	-	-	-	-	-	0.200
Ukrainians	40	-	0.025	-	-	-	-	-	0.300	-	-	-	-	-	-	-	0.444
Iranians	78	-	0.013	-	-	-	-	-	0.026	-	-	-	-	-	-	-	0.231
Israeli Arabs	40	-	-	-	0.025	-	0.025	0.050	0.025	-	0.025	-	-	-	0.025	-	0.200
Israeli Bedouin	32	-	-	-	-	-	0.188	-	0.031	-	-	-	-	-	0.031	0.031	0.219
Palestinians	38	-	-	-	-	-	0.026	-	0.026	-	-	-	-	-	-	-	0.237
Jordanian Bedouin	44	-	-	-	-	-	0.386	-	0.068	-	-	-	-	-	-	0.114	0.273
Kuwaiti	62	-	-	-	0.016	-	0.267	-	0.032	-	-	-	-	-	0.016	0.016	0.133
Saudi Bedouin	40	-	0.025	-	-	-	0.450	0.025	-	-	-	-	-	-	0.025	-	0.150
Syrians	82	-	-	-	-	-	0.037	-	-	-	-	-	-	-	-	-	0.244
Anatolian-Turks	40	-	-	-	-	-	-	-	0.075	-	-	-	-	-	-	-	0.125
Yemeni Hadramaut	156	-	-	0.006	-	-	0.237	-	0.019	0.013	0.006	-	0.032	0.006	0.006	-	0.221
Yemeni Sena	68	-	-	-	-	-	0.294	-	0.147	-	-	-	-	0.059	0.029	0.059	0.294

5.5.2 Population differentiation

Pairwise F_{ST} values were calculated in Arlequin with genotype data for the three sequenced regions and the 2 additional haplotype SNPs. Genotype data for the variant at -13495 were excluded because of too much missing data. Genetic distances between populations are visualised in the PCO plot of Figure 5.3, which shows the first two coordinates as calculated in R.

The Northern European populations are displaced to the right, whereas all other populations are clustered closer together to the left. The Middle Eastern populations are seen together with the Ethiopian and Sudanese samples in the upper part of the cluster. The Asante, Chewa, Chagga and Mambila, which are geographically more distant from Ethiopia and Sudan are located away from the other African populations in the lower part of the plot.

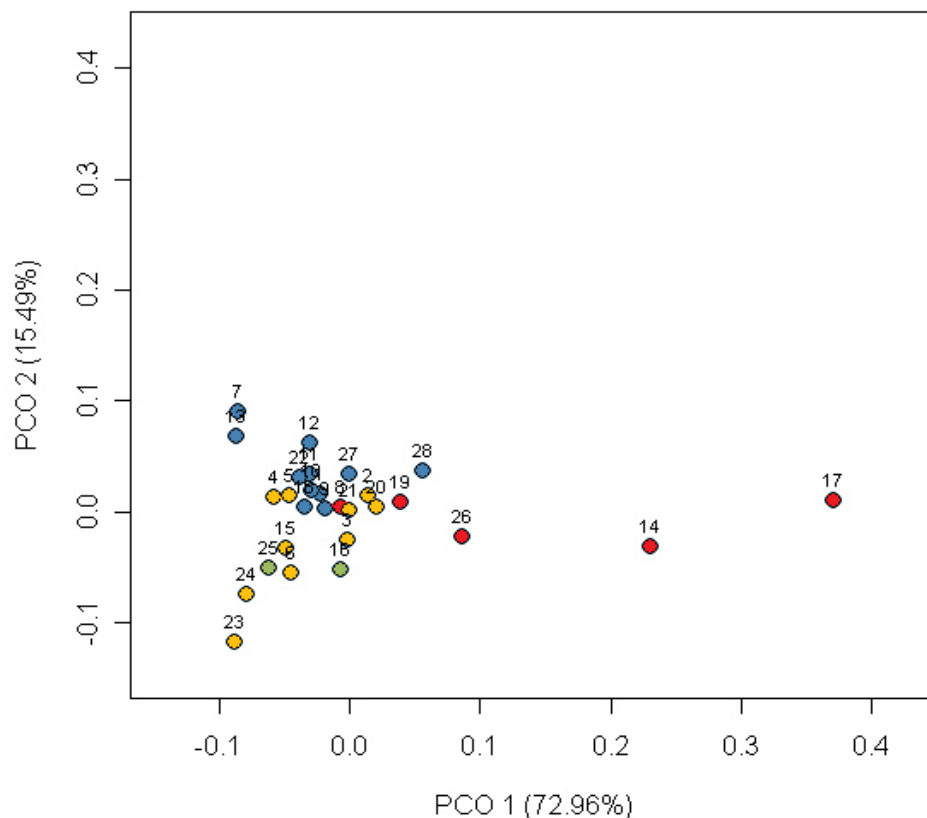


Figure 5.3: Principal co-ordinates plot of genetic distances between populations from pairwise F_{ST} values calculated from genotype data of *LCT* enhancer and the flanking regions. Populations included: 1: Anatolian-Turks, 2: Afar, 3: Shabo, 4: Amhara, 5: Oromo, 6: Asante, 7: Saudi Bedouin, 8: Italians, 9: Israeli Arabs, 10: Israeli Bedouin, 11: Iranians, 12: Jordanian Bedouin, 13: Kuwaiti, 14: Northern Europeans, 15: Chewa, 16: Khalka, 17: Norwegians, 18: Palestinians, 19: Romanians, 20: Beni Amer, 21: Jaali, 22: Syrians, 23: Mambila, 24: Chagga, 25: Tharu, 26: Ukrainians, 27: Yemeni Hadramaut, 28: Yemeni Sena. Colour code: red: European, green: Central Asian, blue: Middle Eastern, yellow: African populations.

5.5.3 Molecular diversity and neutrality tests

Diversity and neutrality measures were calculated using DnaSP with haplotype information of 855 individuals of 28 populations inferred by PHASE (see 5.5.4 below). A combined sequence of the readable sequence parts of 3 regions was compared: 371 bp of *MCM6* intron 4, 559 bp of the *LCT* enhancer region in intron 13 of *MCM6* and 471 bp of the haplotype defining region (hapdef) upstream of *LCT*.

Table 5.5: Haplotype diversity measures for 28 populations across the two control regions and the *LCT* enhancer. N: number of chromosomes, Comp.: Graphic comparison of the regions shows the values for haplotype diversity (Neis' H) for all populations examined. For this analysis -13945 C>T was included as it was possible to infer missing data at that position with PHASE. As previously shown in Jones (2012) but with a shorter enhancer sequence which notably excludes -13495, the non milk drinking African groups (Asante, Chewa, Mambila and Shabo) have a lower heterozygosity level across the enhancer compared to the two flanking regions. However lower heterozygosity of the enhancer can be also seen in some of the European and Asian and Middle Eastern groups as Italians, Romanians, Tharu, Anatolian Turks, Israeli Arabs, Palestinians and Syrians.

A full table with all diversity measures and outcomes of neutrality tests can be found in Appendix C1. Significant values for deviation from neutrality were only obtained for the Israeli Arabs for the enhancer region and the Jordanian Bedouins for the whole sequence for Fu and Li's F statistic and the Yemen Sena for Fu and Li's D for the whole sequence. It is noticeable that values for Tajima's D are usually much lower (although not significantly) in the enhancer than the two flanking regions, except in some European populations such as the Norwegians, Northern Europeans, Ukrainians and Romanians, and also the Beni Amer of Africa.

Table 5.5: Haplotype diversity measures for 28 populations across the two control regions and the *LCT* enhancer. N: number of chromosomes, Comp.: Graphic comparison of the regions.

Region	Population	N	Haplotype diversity (Neis' <i>H</i>) (\pm SD)			
			Intron 4	Enhancer	Hapdef	Comp.
Africa	Afar	124	0.653 (0.000)	0.754 (0.000)	0.644 (0.000)	
	Amhara	80	0.662 (0.000)	0.600 (0.003)	0.661 (0.003)	
	Asante	40	0.714 (0.002)	0.273 (0.008)	0.472 (0.006)	
	Beni Amer	128	0.654 (0.000)	0.786 (0.000)	0.612 (0.001)	
	Chagga	82	0.703 (0.001)	0.459 (0.004)	0.417 (0.004)	
	Chewa	40	0.733 (0.001)	0.273 (0.008)	0.512 (0.002)	
	Jaali	76	0.682 (0.000)	0.752 (0.001)	0.654 (0.001)	
	Mambila	40	0.509 (0.006)	0.355 (0.008)	0.328 (0.006)	
	Oromo	124	0.643 (0.001)	0.598 (0.002)	0.655 (0.000)	
	Shabo	42	0.791 (0.001)	0.297 (0.007)	0.487 (0.003)	
Central Asia	Khalka	38	0.585 (0.005)	0.542 (0.003)	0.519 (0.006)	
	Tharu	36	0.668 (0.003)	0.341 (0.009)	0.595 (0.003)	
Europe	Italians	32	0.752 (0.001)	0.504 (0.006)	0.702 (0.002)	
	N. Europeans	40	0.458 (0.008)	0.522 (0.005)	0.349 (0.008)	
	Norwegians	40	0.229 (0.007)	0.268 (0.007)	0.229 (0.007)	
	Romanians	40	0.713 (0.001)	0.344 (0.007)	0.704 (0.002)	
	Ukrainians	40	0.664 (0.002)	0.627 (0.003)	0.558 (0.004)	
Middle East	Anatolian Turks	40	0.694 (0.003)	0.232 (0.007)	0.679 (0.001)	
	Iranians	76	0.675 (0.001)	0.362 (0.003)	0.674 (0.000)	
	Israeli Arabs	40	0.736 (0.002)	0.555 (0.007)	0.656 (0.001)	
	Israeli Bedouin	32	0.720 (0.001)	0.641 (0.006)	0.677 (0.001)	
	Jordanian Bedouin	44	0.672 (0.001)	0.725 (0.002)	0.707 (0.001)	
	Kuwaiti	56	0.647 (0.002)	0.639 (0.003)	0.662 (0.001)	
	Palestinians	38	0.758 (0.001)	0.422 (0.007)	0.684 (0.001)	
	Saudi Bedouin	40	0.640 (0.002)	0.674 (0.002)	0.653 (0.001)	
	Syrians	82	0.656 (0.001)	0.427 (0.002)	0.680 (0.001)	
	Yemeni Hadramaut	154	0.660 (0.000)	0.675 (0.001)	0.680 (0.000)	
	Yemeni Sena	66	0.716 (0.001)	0.770 (0.001)	0.610 (0.001)	

The comparison of haplotype diversity between lactose digesters and non-digesters of European and African populations shows a clear difference between these populations as well as between the three sequence regions in phenotyped Ethiopians that we have published so far (Jones et al. 2013). Whereas heterozygosity is reduced across the *LCT* enhancer compared to the flanking regions in the non-digester Ethiopian groups, as previously reported, it is *not* different for the Europeans. Furthermore, compared to Africans groups, which show similar diversity in the digesters and non digesters, the Europeans show a higher diversity of the flanking regions in the non-digesters than in the digesters (see Appendix C1). This difference in pattern seems to reflect the two different types of selection acting on that region.

The greater haplotype diversity of the enhancer in the digester Africans than in the non-digesters, is likely to be caused by the co-occurrence of several functional alleles in the digesters due probably to their co-selection (so-called soft selective sweep) but could possibly be due to a reduction in purifying selection. The lower haplotype diversity in the regions flanking the enhancer in the European lactose digesters as compared with the non-digesters instead reflects the much talked about 'hard' selective sweep, caused by increased homozygosity of the extended haplotype carrying the single selected mutation. A full table with results of neutrality tests and diversity measures compared between digesters and non-digesters can be found in Appendix C2.

5.5.4 Haplotype analysis of the *LCT* enhancer and flanking regions

Haplotypes were reconstructed with PHASE from the combined genotype data for the tree sequencing regions (intron 4 and intron 13 of *MCM6* and the hapdef region of the *LCT* promoter) and the two additional haplotype markers 666 G>A and 5579 T>C, spanning a region of about 80 kb, see Figure 5.4. Positions with alleles occurring only once, as well as samples with missing data at more than 4 of the remaining 31 positions were excluded from the PHASE analysis. This left 862 individuals for haplotype analysis, including 7 individuals carrying rare alleles (-14028*C, -14011*T and -13914*A) who were selected for inclusion and did not strictly belong to any of the 28 population samples.

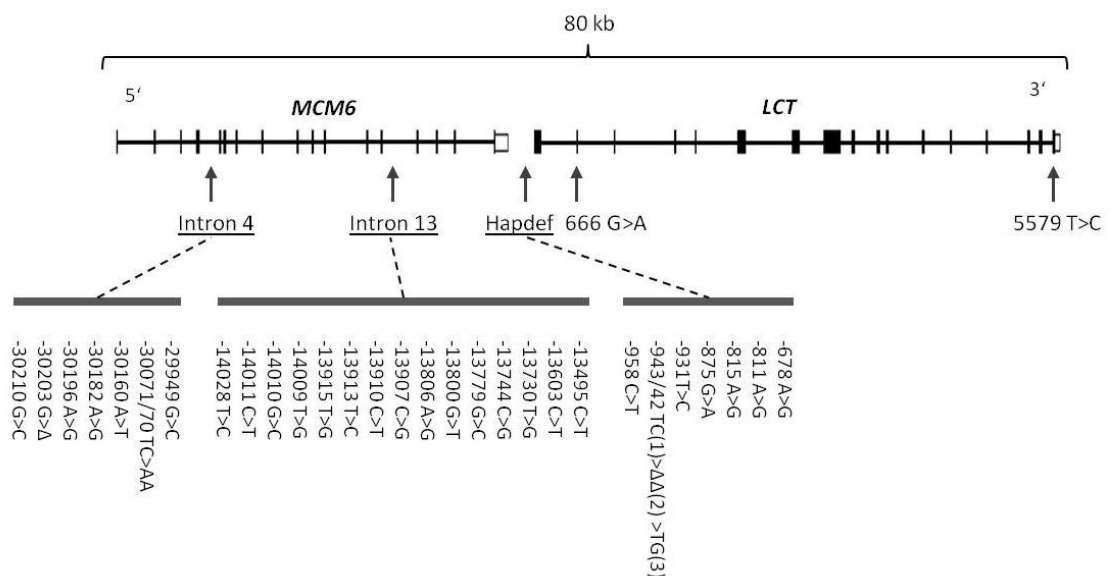


Figure 5.4: Variants included in haplotype analysis of the 80 kb region and their position in relation to the sequenced regions.

Eight of the 11 core haplotype markers, previously defined by Hollox et al. (2001), were used to define the *LCT* haplotype background. One of the set of markers was also the SNP at -946 A>G, but as all samples were homozygous A at that position this was not included in PHASE analysis. The haplotypes inferred by PHASE, especially the rarer ones, were inspected by eye for anomalies and obvious wrong assignments, which left 76 haplotypes (HT) for further analysis. Common haplotypes, linked to the *LCT* core haplotypes, are shown in Table 5.6 and the appearance of the most frequent haplotypes in the populations tested is shown in Table 5.7.

The most frequent haplotype found in this 'global' population is that previously described as B (HT 71 containing -30210*C, which does not carry any derived enhancer allele). The B haplotype is relatively frequent across all four geographic regions but most frequent in the Middle East and Asia and the Afro-Asiatic speaking African groups tested (see Figure 5.5 and Table 5.7). The C haplotype containing the derived allele -13915*G (HT 56, -13915*G, -30182*G) is most common in the Saudi and Jordanian Bedouin and only found in the Middle East, Ethiopia and Sudan whereas the ancestral C haplotype (HT 43, -30182*G) is present in all populations with highest frequencies in the Shabo and Chewa. The ancestral A haplotype (HT 10) carrying the -13495*T allele can be also found across all populations and is most frequent in the Khalka of Mongolia. As expected the -13910*T carrying A haplotype (HT 24, -13910*T, -13495*T) is most prevalent in the Northern Europeans but also present in other European, Asian and Middle Eastern populations, except the Saudi Bedouin and Syrians. In the Africans in this dataset it can be only found in the Jaali, whereas the -13907*G carrying A haplotype (HT 21, -13907*G, -13495*T) is restricted to the Afro-Asiatic speaking African populations and the Yemeni from Hadramaut.

Table 5.6: Inferred haplotypes by PHASE. Low frequency haplotypes (<5) are not shown. Haplotypes in bold are the most probable equivalents (Hollox et al. 2001). N: number of chromosomes. The colour scheme is consistent with the pie charts and network diagram below.

Haplotype ID	-30210 G>C	-30203 G>Del	-30196 A>G	-30182 A>G	-30160 A>T	-30071/70 TC>AA	-29949 G>C	-14028 T>C	-14011 C>T	-14010 G>C	-14009 T>G	-13915 T>G	-13913 T>C	-13910 C>T	-13907 C>G	-13806 A>G	-13800 G>T	-13779 G>C	-13744 C>G	-13730 T>G	-13603 C>T	-13495 C>T	-958 C>T	-943/42 TC>Del>TG (1 TC, 2 DEL)	-931T>C	-875 G>A	-815 A>G	-811 A>G	-678 A>G	666 G>A	5579 T>C	N	Haplotypes
71	C	T	A	.	388	B , G
56	.	.	.	G	G	G	.	.	.	204	C , M, j, k
43	.	.	G	G	.	.	.	200	C , M, j, k
10	T	C	173	A , J
24	T	T	C	110	A , J
7	2	A	.	83	O, S, U , f, g, p
21	G	T	C	63	A , J
31	C	C	56	A , J
66	C	A	.	.	48	P , W, Y
17	G	41	H, K, Q, Z
26	G	2	A	C	33	X , m, n
6	2	A	C	30	X , m, n
42	.	.	G	G	.	C	.	.	27	E
75	C	T	.	.	A	.	.	A	.	.	26	D	
15	T	T	C	19	A , J	
37	T	C	19	A , J	
11	T	17	H, K, Q, Z
82	C	C	A	.	.	14	P , W, Y
49	.	.	G	T	C	14	A , J	
33	C	2	A	C	14	X , m, n
70	C	T	A	C	13	F, I	
53	.	.	G	G	G	.	.	.	12	C , M, j, k
2	C	10	A , J	
80	C	C	T	A	.	.	8	B , G
12	T	A	.	.	6	P , W, Y
30	T	T	C	6	A , J	
52	.	.	G	C	G	.	.	.	5	C , M, j, k	
34	C	2	A	.	.	5	O, S, U , f, g, p
41	.	.	G	5	H, K, Q, Z	

Table 5.7: Distribution of the most common haplotypes across 28 populations studied. Abbreviations for language families: NC: Niger-Congo, AA: Afro-Asiatic, NS: Nilo-Saharan, A: Altaic, IE: Indo-European. N: Number of chromosomes.

Region	Country	Populations	Lang. family	N	71 (B,G)	56 (C, M, j, k) -30182*G, -13915*G	43 (C, M, j, k) -30182*G	10 (A, J) -13495*T	24 (A,J) -13910*T, -13495*T	7 (O, S, U, f g, p)	21 (A,J) -13907*G, -13495*T	31 (A,J) -29949*C	66 (P, W, Y) -30210*C	17 (H, K, Q, Z) -13730*G	26 (X, m, n) -14009*G	6 (X, m, n)
Africa	Cameroon	Mambila	NC	40	-	-	0.200	0.075	-	0.325	-	0.025	0.050	0.075	-	0.150
	Ethiopia	Afar	AA	124	0.274	0.185	0.024	0.065	-	0.024	0.242	-	0.032	0.032	0.008	0.008
		Amhara	AA	80	0.375	0.050	0.138	0.125	-	0.013	0.025	-	0.025	0.050	0.038	0.013
		Oromo	AA	124	0.403	0.145	0.024	0.065	-	0.032	0.048	0.024	0.032	0.024	0.024	0.032
		Shabo	NS	42	0.024	-	0.310	0.071	-	0.071	-	0.143	0.071	0.024	-	0.071
	Ghana	Asante	NC	40	0.100	-	0.175	0.075	-	0.175	-	0.075	0.075	0.075	-	0.050
	Malawi	Chewa	NC	40	0.025	-	0.325	0.100	-	0.125	-	0.050	0.100	0.025	-	0.075
	Sudan	BeniAmer	AA	128	0.125	0.266	0.078	0.047	-	-	0.172	0.008	0.063	0.039	0.133	0.008
		Jaali	AA	76	0.171	0.197	0.118	0.053	0.013	0.013	0.013	0.026	0.039	0.079	0.092	0.026
	Tanzania	Chagga	NC	82	0.085	-	0.146	0.049	-	0.195	-	0.037	0.110	0.049	-	0.049
Central Asia	Mongolia	Khalka	A	38	0.211	-	0.105	0.342	0.026	0.211	-	0.026	-	-	-	-
Europe	Nepal	Tharu	IE	36	0.250	-	0.111	0.111	0.083	0.278	-	0.056	-	-	-	-
Middle East	N. Europeans		IE	40	0.075	-	0.075	0.050	0.650	-	-	0.025	-	-	-	-
	Italy	Italians	IE	32	0.219	-	0.156	0.219	0.031	-	-	0.063	-	-	-	-
	Norway	Norwegians	IE	40	0.025	-	0.100	0.025	0.850	-	-	-	-	-	-	-
	Romania	Romanians	IE	40	0.225	-	0.150	0.050	0.150	-	-	0.025	-	-	-	-
	Ukraine	Ukrainians	IE	40	0.300	-	0.075	0.150	0.300	-	-	0.125	-	-	-	-
	Iran	Iranians	IE	76	0.395	-	0.263	0.145	0.026	-	-	0.026	-	-	-	-
	Israel	Israeli Arabs	AA	40	0.325	0.025	0.100	0.175	0.025	-	-	0.050	0.025	0.025	0.025	-
		Israeli Bedouin	AA	32	0.250	0.188	0.125	0.125	0.031	0.031	-	0.063	0.031	0.031	-	-
		Palestinians	AA	38	0.316	0.026	0.105	0.158	0.026	0.026	-	0.105	0.026	-	-	-
	Jordan	Jordanian Bedouin	AA	44	0.250	0.386	0.023	0.068	0.068	-	-	-	-	-	-	-
	Kuwait	Kuwaiti	AA	56	0.268	0.268	0.161	0.054	0.036	0.036	-	-	-	0.018	0.018	-
	Saudi Arabia	Saudi Bedouin	AA	40	0.150	0.425	0.050	0.100	-	0.025	-	-	0.050	0.025	-	0.025
	Syria	Syrians	AA	82	0.390	0.037	0.122	0.232	-	-	-	0.024	-	-	-	-
	Turkey	AnatolianTurks	A	40	0.375	-	0.150	0.050	0.075	-	-	0.125	-	-	-	-
	Yemen	Yemeni Hadramaut	AA	154	0.182	0.234	0.117	0.136	0.019	0.045	0.013	0.013	0.006	0.006	-	0.006
		Yemeni Sena	AA	66	0.152	0.212	0.061	0.076	0.136	-	-	0.061	-	0.030	-	0.015

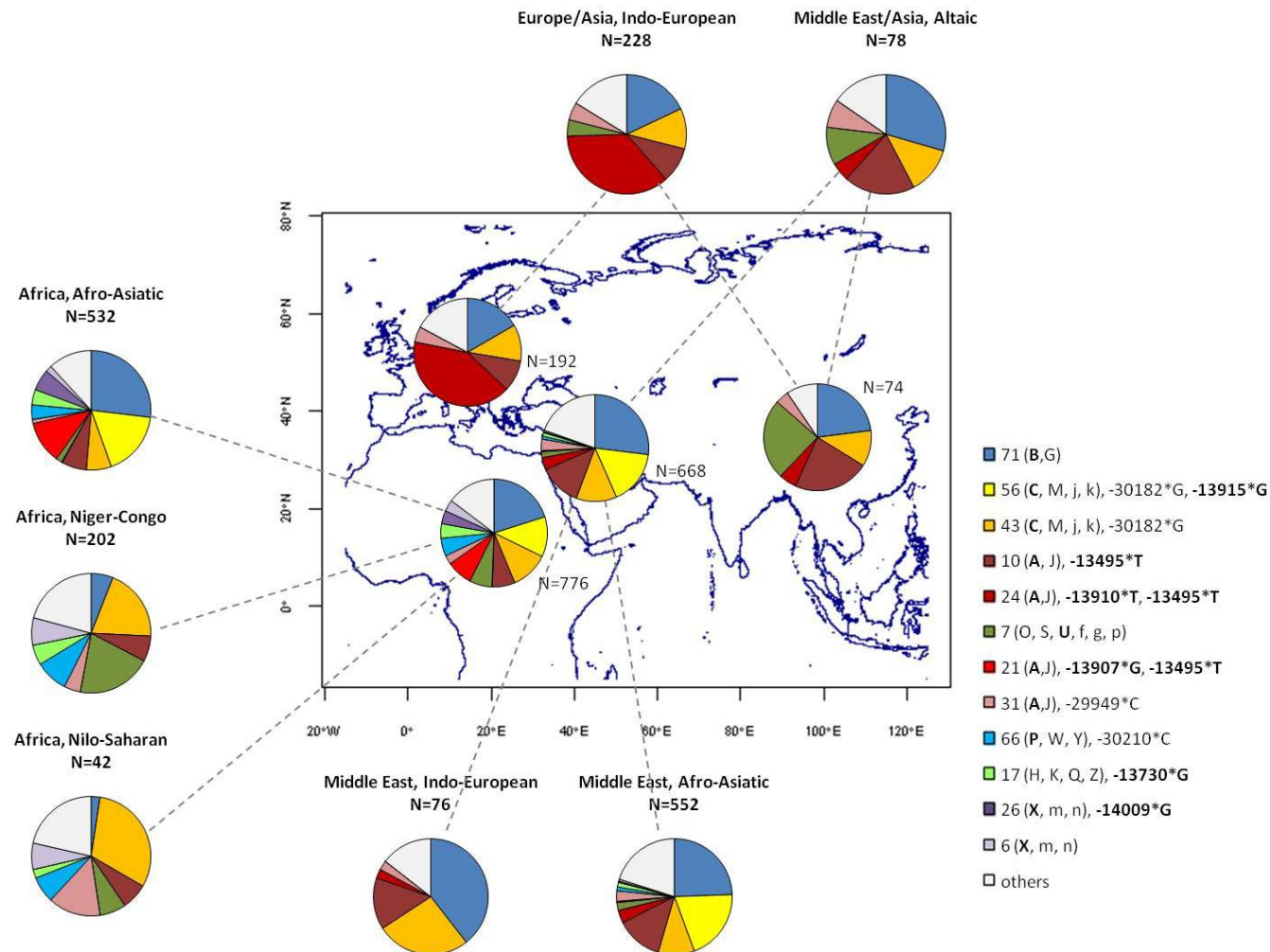


Figure 5.5: Distribution of haplotypes in Africa, Europe, Middle East and Central Asia (within the map) as well as haplotype distribution, classified by language groups. N: Number of chromosomes. Derived enhancer alleles and most probable haplotypes are shown in bold. The colour scheme broadly corresponds to that used in Table 5.6.

5.5.5 Haplotype association of derived *LCT* enhancer alleles

Table 5.6 and Table 5.8 show the relationship of the derived *LCT* enhancer alleles to their haplotype background and to the most common 80 kb haplotypes investigated. Consistent with previous studies (Bersaglieri et al. 2004; Coelho et al. 2005; Mulcare 2006; Poulter et al. 2003) nearly all -13910*T alleles were on the same 80 kb A haplotype (HT 24, -13910*T, -13495*T). Only one *T allele resided on a P haplotype (or W, Y), which is most likely caused by a recombination event between the hapdef region (-678 A>G) and exon 2 (666 G>A) of *LCT*, as the haplotype is the same as HT 24 (A, -13910*T, -13495*T) up to position -678, as indicated in Table 5.8. Myles et al. (2005) found 8 similar cases in Moroccan and Algerian Berber populations.

With the combination of markers used in this thesis it was possible to distinguish between B and P haplotypes and to re-evaluate the occurrence of -14010*C in the previously investigated African samples (Ingram et al. 2009b), as also shown in Jones et al. (2013), and include a further 11 cases. -14010*C exclusively lies on a P haplotype (HT 82) background.

The vast majority of -13915*G alleles have the C haplotype as background (HT 56), which also seems to provide the background for the newly discovered rarer variant -13944*G (HT 59). However in 3 cases -13915*G was found on other haplotypes (E, H and L, as shown in Table 5.8).

Except for three alleles on the ancestral haplotype H, -13907*G is located on an A haplotype background (HT 21), as is -13603*T (HT 15) and most of the -14011*T variants. However two -14011*Ts were found on different B haplotype backgrounds and if the assignments are correct that might suggest this mutation happened several times independently, probably in geographically quite distinct places.

-14009*G mostly occurred on an X haplotype (HT 26) and one on the ancestral H, which is also the background of the majority of the -13730*G variants, though 3 cases were found on A and C haplotypes. The variants -13806*G and -13779*C exclusively occur on the C haplotype background (HT 53 and 52 respectively). -13913*C resides on the B haplotype (HT 80).

The location of *-14028*C*, the variant described in functional studies in the previous chapter, on a B haplotype, was confirmed for both cases. Another rarer variant, *-13800*T*, has U as background haplotype.

To summarise, the derived enhancer alleles in most cases lie on a characteristic haplotype: *-13910*T* on A haplotype as well as *-13907*G* and *-13603*T*, *-13913*C* on a B haplotype, *-13915*G* and *-13806*G* on a C haplotype, *-14010*C* on a P haplotype, *-13730*G* on an H haplotype and *-14009*G* on an X haplotype (Table 5.6 and Table 5.8).

However *-13495*T*, although mostly on A, can in contrast be found on several different haplotype backgrounds, and seems to be evolutionarily much older, and there has clearly been some recombination between it and markers within *LCT* (see also network and LD analyses below).

These results broadly confirm previous findings (Ingram 2008; Jones 2012) but extends them by providing much more information on *-13495*T* and the rare haplotypes, as well as more definitive assignment of *-14009*G* and *-14010*C*.

Table 5.8: Haplotype backgrounds of the lactase persistence associated enhancer alleles. Lactase persistence associated alleles are shown in bold, boxes represent possible recombination events. N: Number of chromosomes. Note that haplotype 25 is like haplotype A for much of the length.

Haplotype ID	-30210 G>C	-30203 G>Del	-30196 A>G	-30182 A>G	-30160 A>T	-30071/70 TC>AA	-29949 G>C	-14028 T>C	-14011 C>T	-14010 G>C	-14009 T>G	-13915 T>G	-13913 T>C	-13910 C>T	-13907 C>G	-13806 A>G	-13800 G>T	-13779 G>C	-13744 C>G	-13730 T>G	-13603 C>T	-13495 C>T	-958 C>T	-943/42 TC>Del>TG (1 TC, 2 DEL)	-931T>C	-875 G>A	-815 A>G	-811 A>G	-678 A>G	666 G>A	5579 T>C	N	Haplotypes		
82	-14010*C																																14	P, W, Y	
	C	1	A	A	A	TC	G	T	C	C	T	T	T	C	C	A	G	G	C	T	C	C	C	C	1	T	G	A	A	A	A	T			
26	-14009*G																																33	X, m, n	
	G	1	A	A	A	TC	G	T	C	G	G	T	T	C	C	A	G	G	C	T	C	C	C	2	T	G	A	A	A	A	C				
27																																	1	O, S, U, f, g, p	
	G	1	A	A	A	TC	G	T	C	G	G	T	T	C	C	A	G	G	C	T	C	C	C	2	T	G	A	A	A	A	T				
56	-13915*G																																204	C, M, j, k	
	G	1	A	G	A	TC	G	T	C	G	T	G	T	C	C	A	G	G	C	T	C	C	C	1	T	G	A	A	G	G	T				
59																																	4	C, M, j, k	
	G	1	A	G	A	TC	G	T	C	G	T	G	T	C	C	A	G	G	C	T	C	C	C	1	T	G	A	A	G	G	C				
55																																	1	E	
	G	1	A	G	A	TC	G	T	C	G	T	G	T	C	C	A	G	G	G	T	C	C	C	1	T	G	A	A	A	G	T				
58																																	1	H, K, Q, Z	
	G	1	A	G	A	TC	G	T	C	G	T	G	T	C	C	A	G	G	C	T	C	C	C	1	T	G	A	A	A	G	T				
57																																		1	L, i
	G	1	A	G	A	TC	G	T	C	G	T	G	T	C	C	A	G	G	C	T	C	C	C	1	T	G	A	A	A	G	T				
24	-13910*T																																	110	A, J
	G	1	A	A	A	TC	G	T	C	G	T	T	T	T	T	C	A	G	G	C	T	C	T	C	1	T	G	A	A	A	G	C			
25																																		1	P, W, Y
	G	1	A	A	A	TC	G	T	C	G	T	T	T	T	T	C	A	G	G	C	T	C	T	C	1	T	G	A	A	A	A	T			
21	-13907*G																																	63	A, J
	G	1	A	A	A	TC	G	T	C	G	T	T	T	C	G	A	G	G	C	T	C	T	T	C	1	T	G	A	A	A	G	C			
20																																		1	A, J
	G	1	A	A	A	TC	G	T	C	G	T	T	T	C	G	A	G	G	C	T	C	C	C	1	T	G	A	A	A	G	C				
22																																		3	H, K, Q, Z
	G	1	A	A	A	TC	G	T	C	G	T	T	T	C	G	A	G	G	C	T	C	T	T	C	1	T	G	A	A	A	G	T			

5.5.6 Haplotype networks

A network was constructed using the Network software and the haplotype information as shown in Table 5.6. The Network is made by assuming single stepwise mutational changes. Figure 5.6 illustrate the relationships of the different haplotypes to each other and locates the derived enhancer and intron 4 alleles of *MCM6*. With more than one appearance of a mutation, recombination can be inferred, as indicated by the ovals. The two exonic SNPs of *LCT* play a large role in haplotype distinction. The network indicates recombination events that could have occurred, especially between the enhancer region and the 5579 SNP in exon 17 of *LCT*, leading to less frequent haplotypes. Most of these events are located between the *LCT* SNPs and position -13495.

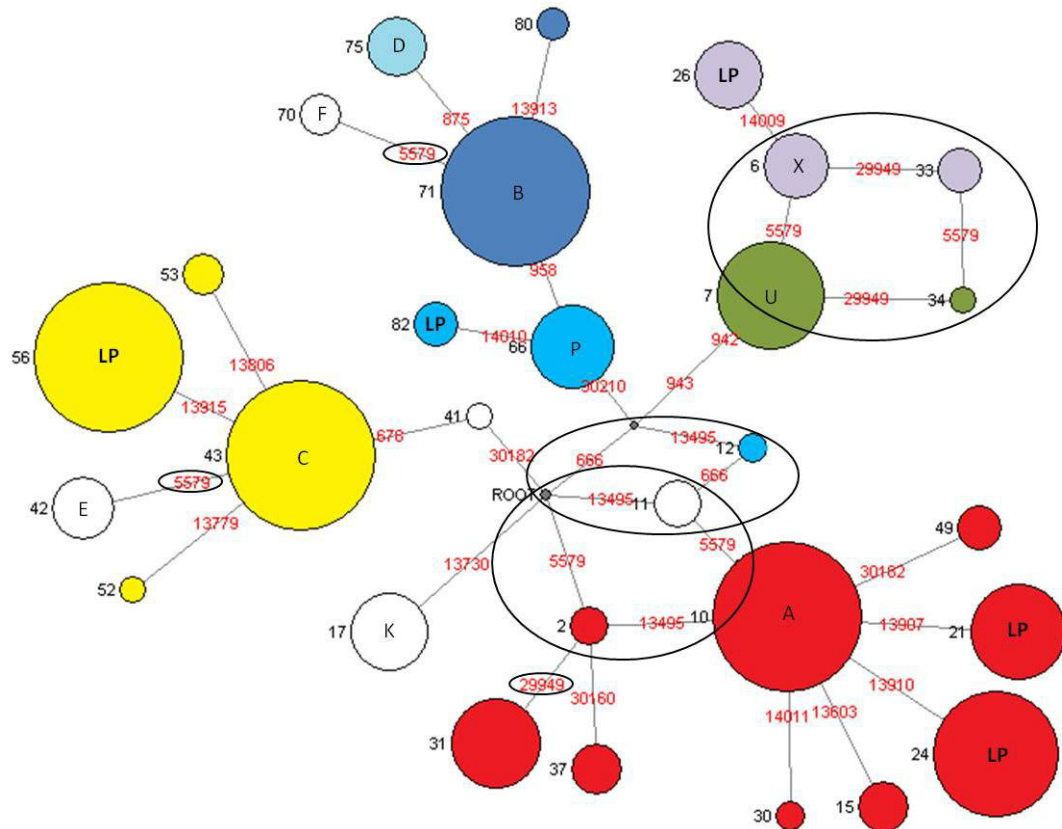


Figure 5.6: Maximum parsimony neighbour joining network of the haplotypes shown in Table 5.6. Haplotype nomenclature corresponds to the haplotype IDs of Table 5.6 and nodes are proportional to haplotype frequency. Names of the most probable *LCT* core haplotypes and those associated with lactase persistence (LP) are indicated, colours represent similar *LCT* core haplotypes and circles indicate recombination events. Note that branch lengths do not represent evolutionary scales.

5.5.7 Linkage disequilibrium

Pairwise linkage disequilibrium across the 80 kb haplotype region was examined using PowerMarker software and values were also checked in DnaSP. The D' measure was calculated with phased haplotype information of 855 individuals. These were grouped in various ways and association patterns differed slightly between the groups examined, partly reflecting the alleles present. An example of the pairwise calculations is shown in Table 5.9, all others in Appendix F.

Linkage disequilibrium was consistently high (D' of 1) between the *MCM6* intron 13 markers, with the exception of the association of -13945 and the two close SNPs at -13907 and -13915 in Afro-Asiatic speaking groups. The lack of statistical significance for the association of some pairs of markers with D' values of 1 (no evidence of recombination) results from low allele frequencies.

A noticeable drop in LD between the 5579 SNP and the markers of the hapdef region is seen in most groups, and also some markers of *MCM6* intron 4. Only in the African groups were there reduced D' values between 5579 and some enhancer variants, probably due to the higher variability of the enhancer in these groups. D' values were lower between -13495 and at least one of the *MCM6* intron 4 SNPS in all language groups.

The Nilo-Saharan population additionally showed reduced D' values between of both exonic markers 5579 and 666 and the -13495 enhancer variant, undoubtedly because the -13495 *T is found on other core haplotypes than A. Both African-Asiatic groups showed a drop in LD between the haplotype markers -958 and -943/942 as well as the Niger-Congo populations between 666 and -943/942.

A more useful graphical way of presenting a picture of historic recombinations is the construction of Linkage Disequilibrium Unit (LDU) maps as was done for extended haplotype analysis as shown below (section 5.5.8.3).

Table 5.9: Pairwise linkage disequilibrium D' across the 80kb haplotype region. Monomorphic markers were excluded from analysis. Statistically significant values after Bonferroni correction for multiple testing for the respective amount of populations per group are shaded in green.

Africa Afro-Asiatic

	-30210	-30203	-30182	-30071/70	-29949	-14010	-14009	-13915	-13913	-13910	-13907	-13806	-13730	-13603	-13495	-958	-943/42	-875	-815	-811	-678	666
-30203	1																					
-30182	1	0.54																				
-30071/70	1	1	1																			
-29949	1	1	1	1																		
-14010	1	1	1	1	1																	
-14009	1	1	1	1	1	1																
-13915	1	1	1	1	1	1	1															
-13913	1	1	1	1	1	1	1	1														
-13910	1	1	1	1	1	1	1	1	1													
-13907	1	1	1	1	1	1	1	1	1	1												
-13806	1	1	1	1	1	1	1	1	1	1	1											
-13730	1	1	1	1	1	1	1	1	1	1	1	1										
-13603	1	1	1	1	1	1	1	1	1	1	1	1	1									
-13495	1	0.14	1	1	1	1	1	1	1	1	0.98	1	1	1								
-958	0.99	1	1	1	1	1	1	1	1	1	1	1	1	1	1							
-943/42	0.89	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.94					
-875	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
-815	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
-811	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1		
-678	1	0.54	0.99	1	1	1	1	1	1	1	1	1	1	0.85	1	0.97	1	1	1	1	1	
666	1	1.0	1	1	0.68	1	1	1	1	1	1	1	1	1	0.93	1	1	1	1	1	1	1
5579	0.96	0.06	0.95	1	0.80	1	0.96	1	1	1	0.93	1	0.86	1	0.78	0.96	0.65	1	1	1	0.95	0.40

5.5.8 Extended haplotype analyses

To investigate how the *LCT* haplotypes carrying the lactase persistence associated enhancer alleles differ from their ancestral forms and how far they expand beyond *LCT* in the combined sample set, additional markers were selected (see 5.4.1). They extend the haplotype analysis across a region of about 1.8 Mb. For 880 individuals 36 markers were genotyped by LGC Genomics. After quality control the genotype data of this set were merged with the data for *MCM6* intron 4 and 13 and the *LCT* hapdef region of the corresponding individuals. All individuals with missing data at more than 6 positions across the whole set or more than 3 positions in the hapdef region, as well as SNPs occurring twice or less were excluded from further analysis. Haplotypes were inferred using PHASE for a final set of 60 SNPs for 837 individuals.

5.5.8.1 Haplotype background the derived enhancer alleles

For comparison the haplotype lengths between chromosomes carrying the ancestral and derived variants on the same haplotype core background A, B, C, P and X (as defined by Hollox et al. 2001) were calculated in bp and plotted.

Figure 5.7 shows all A haplotypes carrying the derived *-13910*T* variant (N=104). The region of about 310 kb is identical for all **T* carrying chromosomes (except one with the ancestral G allele at -22 kb) and spans from SNP rs6430594 located in the *DARS* gene upstream of *LCT* up to the polymorphism rs4954278 in the gene *R3HDM1*.

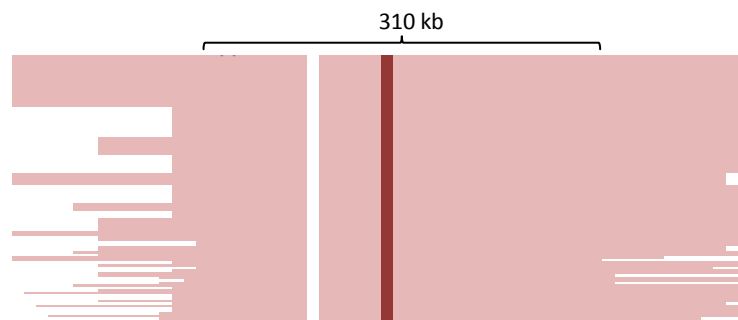


Figure 5.7: Graphical representation of *-13910*T* (red) carrying haplotypes. *-22018*A* (left white bar) is on the same haplotype background. All sequences are identical for at least 310 kb.

As a further example, Figure 5.8 shows the graphical representation of all X haplotypes with ancestral and derived variants at position -14009. The length differences between the derived and ancestral forms can clearly be seen.

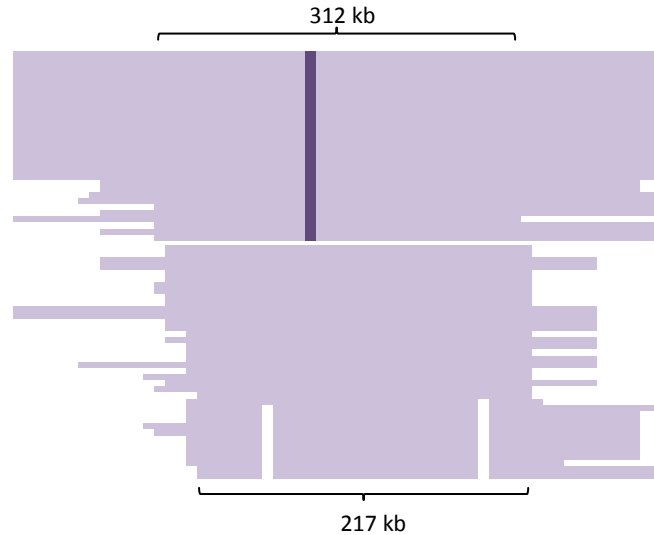


Figure 5.8: Plot of X haplotypes carrying -14009*G (above, with lilac bars) and the ancestral version (below). Kb values represent the minimum haplotype length. The white left bar in the ancestral haplotypes represent -29949*C and the right bar rs2304370, which is located between the *LCT* haplotype defining SNPs at position 666 and 5579. Gene conversion and/or recombination can be inferred for these haplotypes particularly from -29949 upstream which would shorten the minimum length of the ancestral haplotype to 55 kb and the mean to 655 kb.

The mean lengths were calculated for all haplotypes carrying the derived allele versus all others in calculating the haplotype length of each chromosome as sequence region shared with the most frequent haplotype (for example shown as broad upper bar in Figure 5.7), and results are shown in Table 5.10. For comparison of each of the two groups, a two tailed Mann-Whitney U test was performed. The mean lengths of the haplotypes carrying the derived lactase persistence associated alleles are significantly longer ($p < 0.001$) than those carrying the ancestral alleles on the SNP positions.

Interestingly, the ancestral B haplotype is also relatively long and the -13913*C variant, which is not associated with lactose digester status, also occurs on a slightly longer B haplotype, though this difference is not statistically significant ($p = 0.68$). This smaller difference presumably indicates that this non-functional mutation is much older than the others.

Table 5.10: Mean haplotype length of core haplotypes carrying derived and ancestral alleles.

Haplotype	variant	Haplotype length (kb)	
		derived	ancestral
A	-13910*T	1342	658
	-13007*G	1580	
C	-13915*G	1473	549
X	-14009*G	1578	675
P	-14010*C	1417	849
B	-13913*C	1076	1004

5.5.8.2 Extended haplotype homozygosity (EHH)

Previous studies of long range haplotypes across the *LCT* region were mainly done considering the -13910 C>T to be functional for lactase persistence (Bersaglieri et al. 2004; Sabeti et al. 2007). These and other lactase persistence associated alleles were compared to all haplotypes carrying the ancestral variant at that position (Enattah et al. 2008; Tishkoff et al. 2007), i.e. all other *LCT* core haplotypes combined together. In a more recent study we conducted a long range haplotype test on the A carrying 13910*T haplotype in comparison with ancestral A, B and C (Gallego Romero et al. 2012). Remarkably, the B haplotype carrying none of the associated alleles showed a long stretch (500 kb) of relatively high extended haplotype homozygosity (EHH).

To investigate selection patterns in the region around *LCT* in the combined dataset, EHH tests were first conducted using the Sweep software for the different lactase persistence associated alleles as reported previously by others, using the single derived allele as core and the EHH values plotted in R (see also section 2.3.6 of chapter 2). Figure 5.9 to Figure 5.11 show the plots of for the decay of EHH against genetic distance from the core SNPs. It should be noted that all plots illustrate are shown in the chromosomal + direction, as also in Figure 5.2, which is the opposite way round to the previous graphs and tables of this chapter, which are shown in the direction of orientation of the *LCT* and *MCM6* genes.

Haplotypes carrying the derived lactase persistence associated alleles were analysed against all other haplotypes of the whole sample set, except for -14009*G and -14010*C where analyses were done on a subset of samples because of their low frequency in the overall sample set, on the African Afro-Asiatic speaking (522 chromosomes) and Niger-Congo speaking (202 chromosomes) groups respectively. To get more information about the B haplotype the same was done for the -958 SNP as core (Figure 5.10).

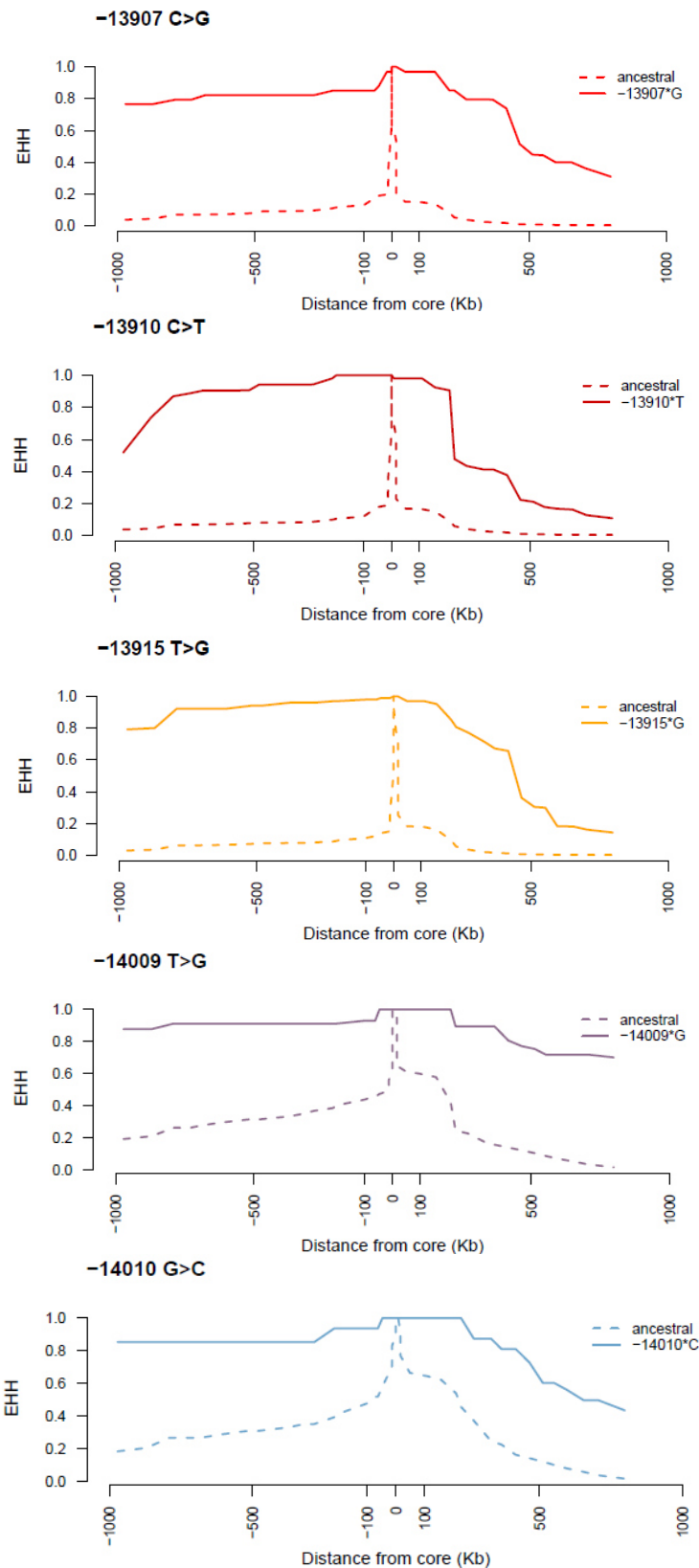


Figure 5.9: Decay of extended haplotype homozygosity for the haplotypes carrying derived LP associated alleles of the *LCT* enhancer compared to those carrying ancestral alleles. For variants at -13907, -13910 and -13915 these were compared to all other haplotypes of the set of 1674 chromosomes and for -14009 and -14010 for a subset of 522 and 202 chromosomes respectively.

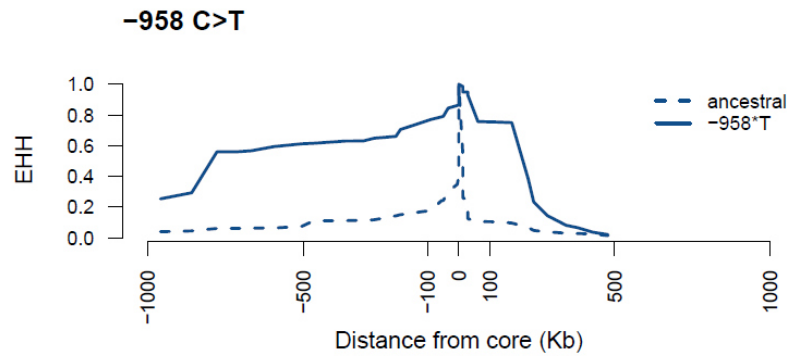


Figure 5.10: EHH decay of the haplotypes carrying -958*T (as key marker for the B haplotype) against all other chromosomes of the whole sampleset (n=1674).

All haplotypes carrying the derived enhancer alleles show greater EHH on both sides of the core SNPs in comparison to all other haplotypes (Figure 5.9 and Figure 5.10). This shows evidence for recent origin of these alleles and is also consistent with selection of these variants, and is consistent with previous findings for some of the variants (Bersaglieri et al. 2004; Gallego Romero et al. 2012; Poulter et al. 2003; Sabeti et al. 2007; Tishkoff et al. 2007; Voight et al. 2006). It should be noted that the EHH decays in a non-symmetrical manner. It drops more quickly on the right side of core SNP on the chromosome (i.e. upstream of the enhancer in *LCT* gene direction, chromosome -), which is particularly apparent for the -13910*T carrying haplotype. It is also of interest that this same extended haplotype homozygosity is detectable for -13907*G,, which is located on the same haplotype background, even though there are more 13910*T alleles in the dataset.

To be able to compare between the *LCT* core haplotypes with the derived or ancestral alleles of the lactase persistence associated enhancer SNPs, the A, C, P and X haplotype chromosomes were selected from the data and EHH tests done for each of them separately but the results combined as shown in Figure 5.11. In addition to that the B haplotype chromosomes were selected from the overall dataset and setting -958 as core SNP against all other preselected chromosomes. In this comparison -958*T alleles carried on derived and recombinant chromosomes D, F, I, R, N, a, L, i and B -13913*C are excluded.

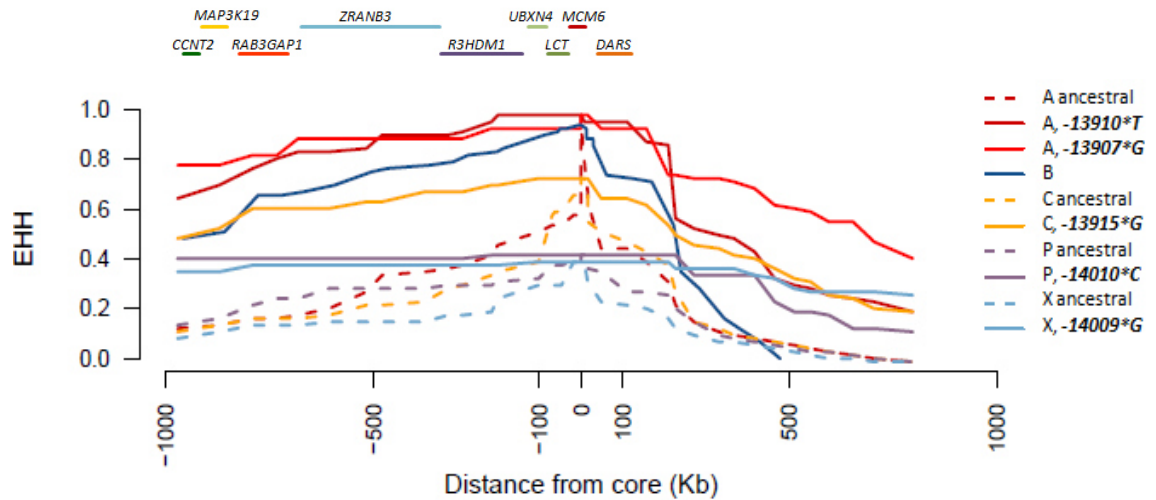


Figure 5.11: EHH decay over physical distance for the haplotypes carrying derived LP associated alleles compared to their ancestral haplotypes and all B haplotype carrying chromosomes. Note that the derived haplotypes for P and X represent less frequent chromosomes of $n=13$ and $n=31$ respectively. The broad location of the genes on the chromosome is indicated above the plot.

A large step in EHH decay is located about 230 kb chromosomal upstream of the core SNPs (i.e. to the right hand-side on the diagram on Figure 5.11), which is similar for all variant haplotypes. To the other side, the haplotypes show a long stretch of EHH at relatively high levels, with a small step of decay for the -13910^*T and -13907^*G carrying haplotypes far beyond *LCT* in gene *R3HDM1* and further away about 800 kb in gene *RAB3GAP1*.

Interestingly this long stretch of EHH can also be seen in the B haplotypes that do not carry any of the functional derived alleles. Even in Figure 5.10, where only the SNP at -958 distinguishes between the haplotypes, a stretch of about 500 kb can be seen in about 60% of the chromosomes. Note that in this case of the -958^*T carrying haplotypes that the small sharp drop in EHH close to the core SNP is probably mainly due to the recombination event, which leads to the F haplotype (see Figure 5.6).

The critical question is whether this pattern is likely to be caused by selection of another allele outside of the enhancer but within the 500 kb region, or other factors. There is clear evidence that this haplotype is old since it occurs on many continents.

From the Genome Browsers the recombination rates in the tested genomic region are shown to be relatively low. The estimated recombination rates between the regions 135-136 and 136-137 Mb of chromosome 2 show only a minor difference of 0.5 cM/Mb and 0.4 cM/Mb respectively (deCODE sex averaged rates, 0.4 and 1.1 Marshfield and 0.8 both Genethon sex averaged rates respectively, UCSC genome browser) but increase to 0.7 (2.0

and 1.7 respectively) from 137 Mb upstream. Linkage disequilibrium maps were therefore used to get further information about recombination patterns of the examined 1.8 Mb region in different HapMap populations.

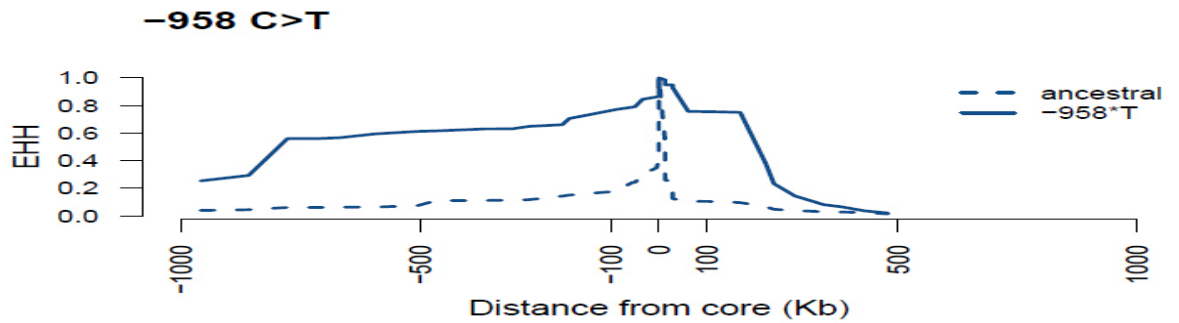
5.5.8.3 Linkage Disequilibrium Unit (LDU) maps

The construction of LDU maps is based on the idea that LD decays with distance and pairwise LDU for each SNP are calculated using $\epsilon_i d_i$, the product of the exponential decline ϵ of association between SNPs and their distance d in kb (Maniatis et al. 2002, see also chapter 2, 2.3.5). The information is combined over many adjacent SNPs sequentially and converted to LD units. These units are plotted against distance resulting in a graph that shows a series of steps, where the steps in the line represent the breakdown in LD due to recombination and the plateaus high LD. LD units can also be plotted as LDU/kb against distance to get a series of peaks, which also represent recombination hotspots. With the help of Winston Lau this has been done on publically available Hap Map Phase III data of release 28.

From Figure 5.12 it can be seen that the patterns of LD and areas of a breakdown of linkage are similar in all populations. Where the data are plotted to show LDU/kb against kb and it can be seen more clearly that the peaks that represent recombination hotspots are also similar. A long stretch of no recombination across *ZRANB3* can clearly be seen in all populations in these plots, irrespective of whether they are known to have frequent LP alleles. This extends in the CEU population until *MAP3K19*. In the other two populations there is a small amount of recombination between *ZRANB3* and *MAP3K19*. The region upstream of *LCT* including *MCM6* and *DARS* also shows relatively low recombination, with only a small peak between *MCM6* and *DARS* in the African in European populations. Interestingly, there is a high peak of recombination within *LCT*, at the 3' end of the gene, in the Chinese, which is lower for the other populations and which could reflect the region around the 5579 SNP.

Even more noteworthy is the observation that the LDU maps show considerable correspondence to the EHH plots for the derived alleles and for the B haplotype (Figure 5.12). These show a large region of haplotype homozygosity that stretches along *ZRANB3* partly including *RAB3GAP1* and *R3HDM1*. On the other side, beyond *DARS*, haplotypes seem to break down, which is similar to the LDU plots. Figure 5.13 shows the same plot more focussed on the genotyped region.

a)



b)

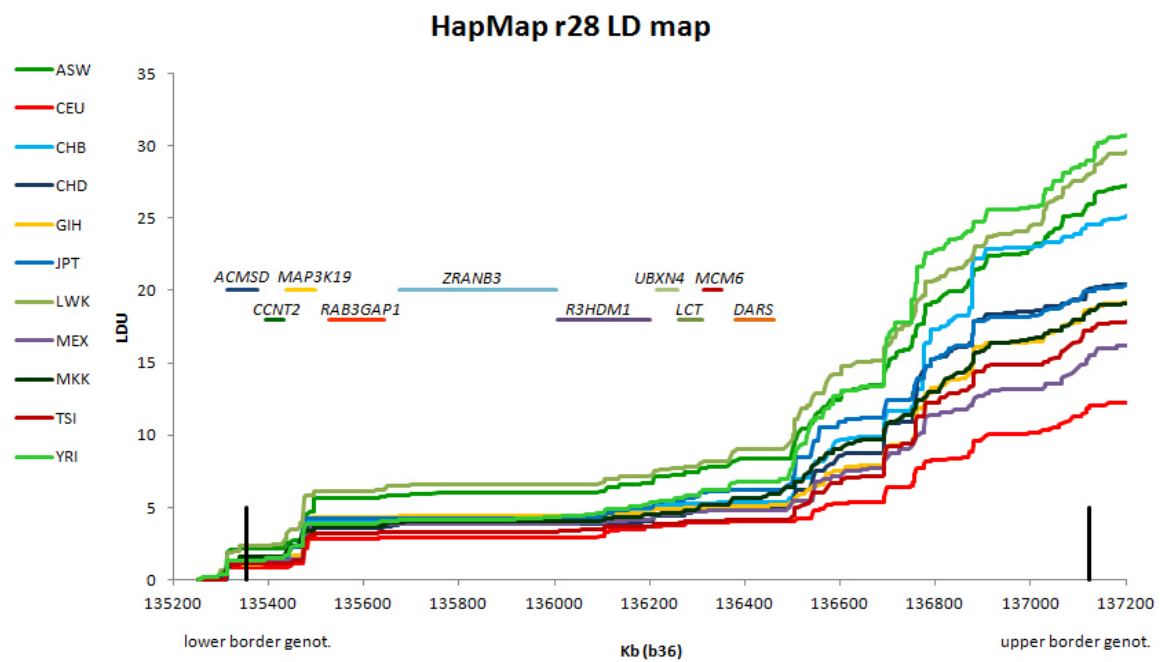


Figure 5.12: Linkage disequilibrium map showing LDU against distance (b) in comparison to the EHH plot of -958 C>T (a). The borders on the x-axis of the LD map represent the region genotyped for the samples in this chapter. The colour codes represent different HapMap populations: ASW: South western Americans with African ancestry, CEU: CEPH, Utah residents with Northern and Western European ancestry, CHB: Han-Chinese from Beijing, CHD: Chinese from Denver, GIH: Gujarati Indians from Houston, JPT: Japanese, LWK: Luhya from Webuye, Kenya, MEX: Mexican ancestry from Los Angeles, MKK: Maasai from Kinyawa, Kenya, TSI: Toscani from Italia, YRI: Yoruba from Ibadan, Nigeria.

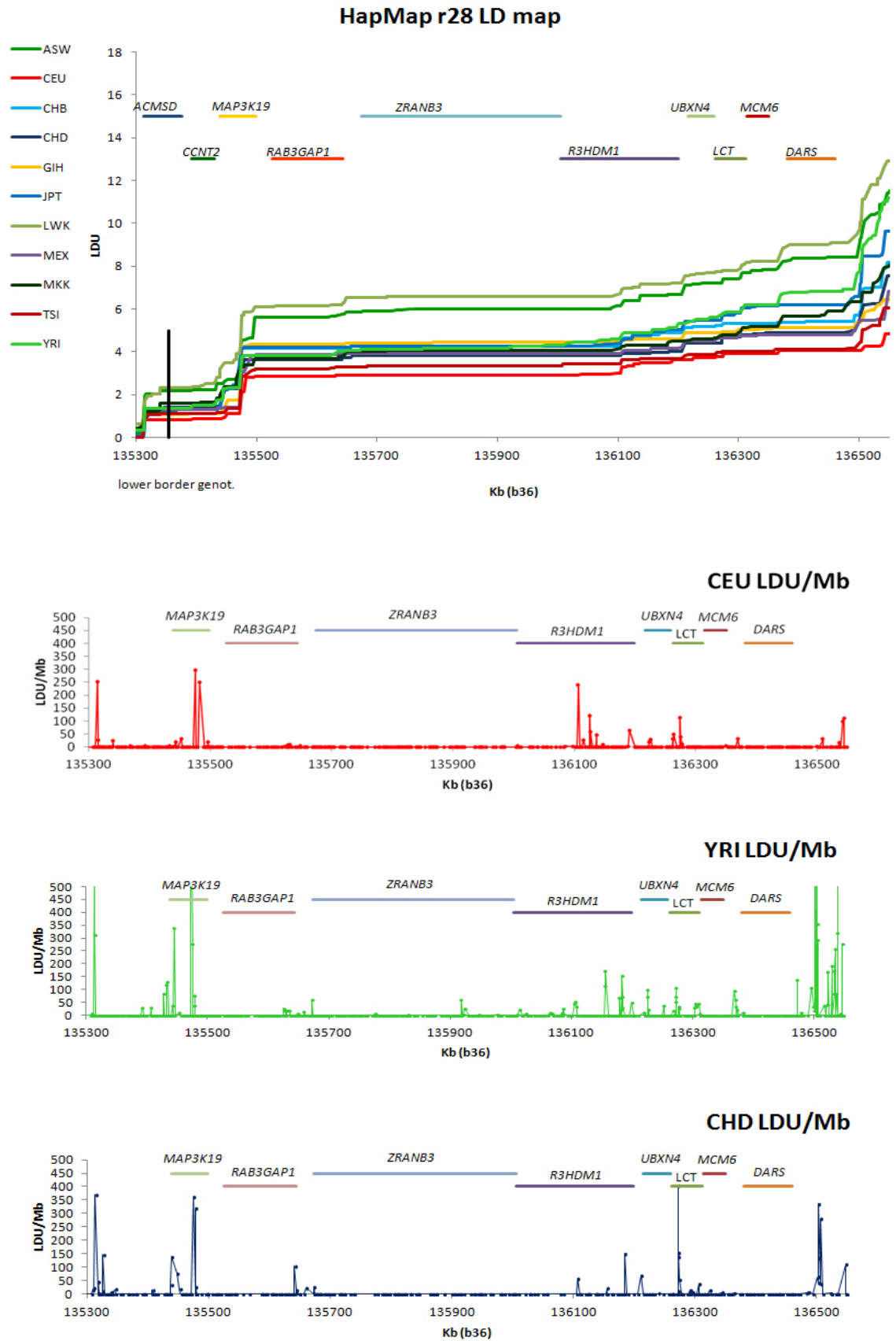


Figure 5.13: LDU against distance plot (upper plot) for the genetic region included in extended haplotype analysis and corresponding LDU/Mb against distance plot for several HapMap populations (abbreviations in legend Figure 5.12) showing peaks of recombination hotspots.

5.6 Discussion

In this chapter genotype and haplotype data from a wide set of samples from different continents were collected and interpreted to get further insights into the evolution of the *LCT* enhancer alleles and detect possible signatures of selection. Different approaches were used to examine the *LCT* haplotypes.

The analysis of the 80 kb haplotype around *LCT* allowed the construction of a Network and confirmed a tight association of the lactase persistence associated enhancer variants with particular haplotypes as described previously (Enattah et al. 2008; Ingram 2008; Ingram et al. 2007; Tishkoff et al. 2007). The patterns of LD and haplotype diversity show differences in the various populations, with the least diversity in Northern Europe. Comparing the *LCT* enhancer region with the two regions upstream and downstream, shows a reduction in diversity of the flanking regions in lactose digesters in comparison with non-digesters, unlike the situation in Africans where the digesters show equivalent or more diversity in the enhancer. This pattern reflects the different consequences of positive selection for one allele on a single haplotype background, and several alleles on different haplotypes (e.g. Bersaglieri et al. 2004; Ingram et al. 2009b; Jones et al. 2013).

The high frequency and wide distribution of the B haplotype, also shown in other studies (Gallego Romero et al. 2012; Hollox et al. 2001; Ingram et al. 2007; Jones et al. 2013) was confirmed in the combined dataset, and its appearance across the continents suggests that this is an old haplotype.

With the extension of the haplotype analysis to about 1.8 Mb it was possible to look further for possible signatures of selection for the lactase persistence associated alleles in the dataset. Different approaches were used, which came to similar conclusions. It was shown that the haplotypes carrying the derived lactase persistence associated alleles are much longer than their ancestral haplotypes, as calculated from their mean haplotype length. This pattern could also be shown with EHH analysis. The extended haplotype homozygosity of the lactase persistence haplotypes spans longer regions compared to all other haplotypes of the datasets as well as the haplotypes on which they arose. These differences support the recent dating of these variants with lack of time and therefore recombination events to occur and break them up (Sabeti et al. 2006). Proper examination of their frequency in less heterogeneous datasets would be likely to confirm a signature of selection by methods used by others (Sabeti et al. 2006; Voight et al. 2006). However,

aside from possible sampling issues, this chapter highlights one problem with the analyses reported so far. That is that the comparison of a lactase persistence associated allele carrying haplotype against all other haplotypes somewhat exaggerates this effect, because the non-carrier chromosomes include a mixture of chromosomes of different the *LCT* core haplotype.

The analysis of derived and ancestral variant haplotypes of the same *LCT* core haplotype (as first shown by Gallego Romero et al. 2012) seemed to be a better way of representing the effect of a certain allele. The haplotype lengths of all chromosomes with an A, B, C, P and X haplotype backgrounds were calculated from all phased chromosomes clustered using the haplotype definition of Hollox et al. (2001) and were shown to be significantly different for the functional enhancer alleles. Results from EHH analysis revealed longer stretches of haplotype homozygosity for the lactase persistence variant alleles than their ancestral alleles (only shown visually). However, extended haplotype homozygosity was also observed for the B haplotype, which does not carry any functionally important enhancer alleles. This is a pattern that had been noticed before (Gallego Romero et al. 2012).

Regions of LD as seen in the EHH plots interestingly overlap with those from LDU plots for different populations and are similar for all haplotypes. LD decays rapidly upstream of *LCT* (downstream in chromosomal direction), after *DARS* (aspartyl-tRNA synthetase). However, the region of LD to the other side of *LCT* is much longer and spans across *ZRANB3* (zinc finger, RNA-binding domain containing 3), up to *MAP3K19* (Mitogen-activated protein kinase kinase 19), where a decay of LD can be seen.

The LDU plots show that *ZRANB3* region has not been disrupted by recombination. This might somehow have been an effect of selection for reduced recombination due to its functional importance. *ZRANB3* was shown to be a DNA annealing helicase and endonuclease with function for genome stability (UniProtKB, <http://www.uniprot.org/>). It is important for replication stress response and was for example shown to be involved in the repair of DNA lesions that block the replication process (Weston et al. 2012). Whether or not as a consequence of selection affecting this or another gene, it may be that there is a lack of recombination motifs (Myers et al. 2010) or even a structural rearrangement of the chromosome in this region. Such repression of recombination will have distorted inferences about hard selective sweeps.

From the combined results of this chapter it can be concluded that all *-13910*T alleles* occur on one haplotype which extends at least 310 kb and in many cases much longer (mean length 1.3 Mb). This supports the conclusion of previous findings which suggest one single origin of *-13910*T* and its presence on one selected haplotype (for example Bersaglieri et al. 2004; Poulter et al. 2003). Our failure to find *-13910*T* on any different background even in Iran does not provide support for the existence of a convergent evolution of the allele as claimed previously (Enattah et al. 2007). Inspection of the haplotype networks in that paper make errors in haplotype inference or gene conversion more likely, as the 22kb SNP occurred on two different branches of the network.

The same pattern of extended haplotypes is also seen for all other variants that have been shown to be functional, apart from -14011 C>T, for which there is some break-up of haplotype background. It is of interest to note that in the case of *-13913*C*, for which there is strong evidence that it does not cause lactase persistence, the derived allele is also located on a long haplotype in the 7 chromosomes that we had to analyse, but this is not significantly longer than that of the ancestral B variant on which it arose, even though its low frequency would suggest a very recent origin. So in that case one might speculate that the allele was spread by drift.

The observations on the extended region of high LD in all populations, including many without LP and of the extended B haplotype, show that new methods ought to be developed to examine selection to take demography and recombination into greater account

6 LCT immediate promoter and intron 2 - a search for yet undiscovered functional variants

6.1 Introduction

The genotype-phenotype comparison maps in chapter 3 show areas where lactase persistence frequencies of all 5 variants thought to be causal of the trait, combined, fails to match the observed phenotype data. Besides sampling issues and incomplete datasets of genotype- or phenotype data these interpolated maps show geographic candidate regions where other causes of lactase persistence might yet be uncovered. One example for missing lactase persistence alleles, as already mentioned in chapter 1, comes from the unexpected lack of enhancer alleles in the Wolof (Ingram et al. 2009b; Jones et al. 2013; Mulcare et al. 2004) with half the sample tested being judged lactase persistent by lactose tolerance tests (Arnold et al. 1980).

With the discoveries of the last years, revealing more lactase persistence variation in the enhancer region of *LCT*, the doubts about this region being of critical functional importance have subsided, but speculations remain that other causes for lactase persistence exist and it cannot be excluded, and it is indeed likely, that other sequence regions influence lactase expression.

A recent reinvestigation of the exonic region around the active sites of lactase in Africans did not reveal any variation (Jones 2012) which might have altered lactase expression and all lines of evidence pointed to regulatory variation being more likely to be responsible for inter individual difference in adult lactase expression. In an effort to find other variation of possible function, a study at the outset of this thesis work showed variation in the immediate promoter of the lactase gene in several African individuals, which was slightly surprising since this region had been shown to be a quite conserved part of the gene (Troelsen 2005). Two alleles, -17^*T and -36^*T (rs111985625), were of interest as they were only found in individuals from milk drinking populations (Jones 2012).

6.2 Chapter aims

These new findings inspired the examination of the immediate promoter region in further samples of European and Middle Eastern origin. A further aim of this chapter was to use bioinformatic tools and publicly available databases to determine new genomic regions in and around the *LCT* gene with a possible functional influence on lactase persistence. One such region would be selected and examined. Depending on the results *in vitro* experiments would be designed to examine the effect of nucleotide changes in one or both regions.

6.3 Strategy

For the promoter region of *LCT* previously tested primers were used (Jones 2012) to amplify a 675 bp sequence region (Chr2:136594559-136595233, GRCh37/hg19) of which a sequence of bp -360 until +82 from the *LCT* transcription start was readable for all samples. Details of primer sequences are shown in Table 2.3, chapter 2.

To identify candidate regions in and around *LCT* with possible function the UCSC genome browser (<http://genome.ucsc.edu>) was scanned in particular for bioinformatically identified transcription factor binding sites and conserved regions between species. Especially useful was information from two publications that used a combination of chromatin immunoprecipitation and next generation sequencing (ChIP-Seq) to identify potential Cdx-2 and HNF-4 α binding sites in Caco-2 cells (Boyd et al. 2009; Boyd et al. 2010). As mentioned in chapter 4, both transcription factors play an important role in the intestine. One region in intron 2 of *LCT* showed high binding affinity to both transcription factors in these assays (as shown in Figure 6.1) and was chosen for further investigation via sequencing.

Primers were designed to include the most of the sequence region of interest. With these primers a region of 839 bp was amplified (Chr2:136588631-136589469, GRCh37/hg19) of which bp positions 5345-6052 of *LCT* were readable.

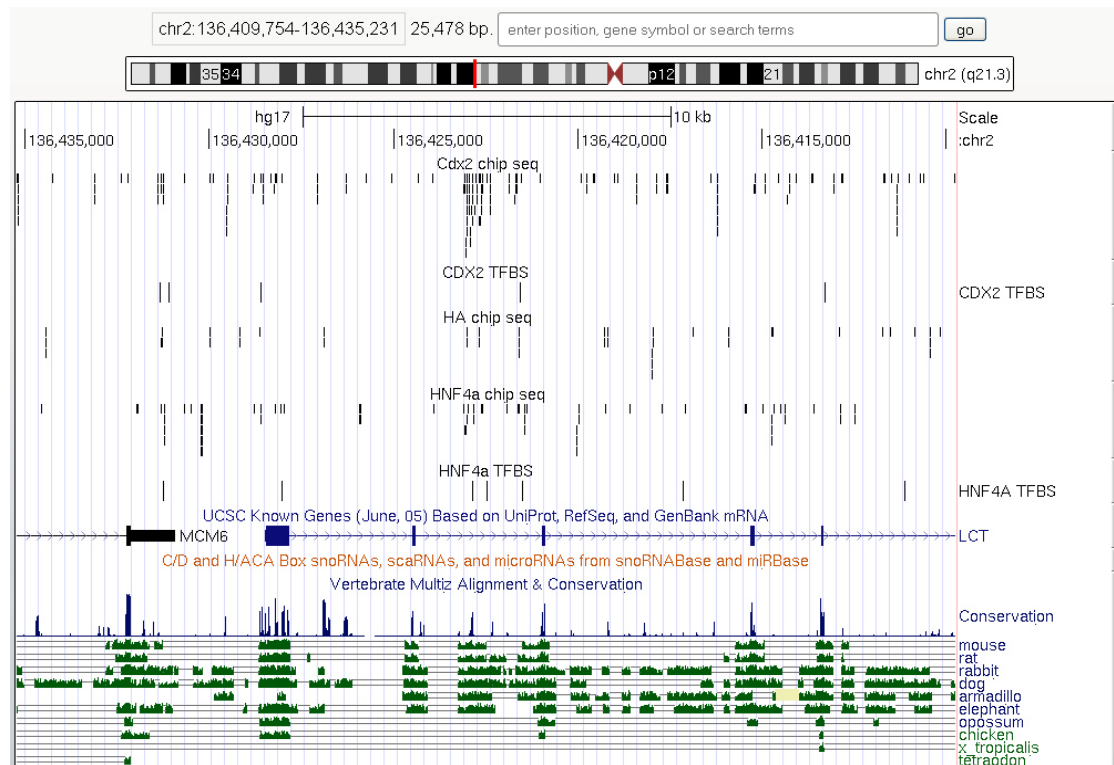


Figure 6.1: Output of the UCSC Genome Browser of the human sequence (<http://genome.ucsc.edu>, NCBI35/hg17) showing the positions of Cdx-2 and HNF-4 α binding in intron 2 of *LCT* in ChIP-Seq assays (Boyd et al. 2009; Boyd et al. 2010).

6.4 Variation of the immediate *LCT* promoter

The immediate promoter was successfully sequenced for 1018 individuals from 45 populations. Table 6.1 shows all variants found in the *LCT* promoter region. 6 new variants were identified (-333 C>T, -294 C>A, -237 G>A, -211 C>T, -204 C>T, 41 T>C) most of which are rare. Only -294*A, -211*T and -17*T occur in more than one population. Of the more frequent African alleles (Jones 2012) -173*A was found in two Yemeni samples from Hadramaut and -17*T in 6 populations of which most are also from the Middle East.

Table 6.1: *LCT* promoter variation (allele counts) in the samples sequenced from 45 European and Asian population groups, N: number of chromosomes.

Region	Country	Population	N	-333 C>T	-294 C>A	-237 G>A	-211 C>T	-204 C>T	-173 G>A	-17 C>T	41 T>C
Northwest/ Central Europe	Germany	Germans	60	-	-	-	-	-	-	-	1
		Sorbs	64	-	-	-	-	-	-	-	-
	Ireland	Irish	68	-	-	-	-	-	-	-	-
	Netherlands	Frisians	54	-	-	-	-	-	-	-	-
	Norway	Norwegians	54	-	1	-	-	-	-	-	-
	Slovakia	Roma	64	-	-	-	-	-	-	-	-
	Sweden	Sami	10	-	-	-	-	-	-	-	-
		Swedes	14	-	-	-	-	-	-	-	-
	UK	Ashkenazi-Jews	4	-	-	-	-	-	-	-	-
		English	52	-	-	-	-	-	-	-	-
South Europe	Other C/NW Europeans		44	-	-	-	-	-	-	-	-
	Greece	Greeks	40	-	-	-	-	-	-	-	-
	Italy	Tyroleans Bozen	62	-	-	-	-	-	-	-	-
		Tyroleans Gadertal	58	-	-	-	-	-	-	-	-
		Tyroleans Vinschgau	60	-	-	-	-	-	-	-	-
	Portugal	Portuguese	38	-	-	-	-	-	-	-	-
	Spain	Catalans	60	-	-	-	-	-	-	-	-
		Spanish	62	-	-	-	-	-	-	1	-
East/ Southeast Europe	Belarus	Belarusians	44	-	-	-	-	-	-	-	-
	Macedonia	Macedonians	64	-	-	-	-	-	-	-	-
	Romania	Romanians	64	-	-	-	-	-	-	-	-
	Russia	Erzja	2	-	-	-	-	-	-	-	-
		Russians Perm	34	-	-	-	-	-	-	-	-
	Ukraine	Ukrainians	28	-	-	-	-	-	-	-	-
	Other E/SE Europeans		12	-	-	-	-	-	-	-	-
Middle East	Cyprus	Greek Cypriots	50	1	-	-	-	-	-	-	-
	Iran	Iranians	90	-	1	-	-	-	-	-	-
	Kuwait	Kuwaiti	50	-	-	-	3	-	-	-	-
	Syria	Syrians	114	-	-	-	-	1	-	2	-
	Turkey	Anatolian-Turks	40	-	-	-	-	-	-	-	-
	Yemen	Yemeni Hadramaut	134	-	1	-	-	-	2	2	-
		Yemeni Sena	28	-	4	-	-	-	-	1	-
	Other Middle Easterns		48	-	-	-	-	-	-	1	-
West Asia	Armenia	Armenians	50	-	1	-	-	-	-	-	-
	Azerbaijan	Azeri	50	-	-	-	1	-	-	-	-
	Georgia	Georgians	38	-	-	-	-	-	-	1	-
Central/ South Asia	Afghanistan	Pashtuns/Afghans	32	-	-	-	-	-	-	-	-
		Tadjiks	12	-	-	-	-	-	-	-	-
		Uzbeks	12	-	-	-	-	-	-	-	-
	Nepal	Nepalese	10	-	-	-	-	-	-	-	-
		Tharu	42	-	-	-	-	-	-	-	-
	Uzbekistan	Uzbeks	42	-	-	1	-	-	-	-	-
	Other C/South Asians		14	-	-	-	-	-	-	-	-
Central/East/ Southeast Asia	Mongolia	Khalka	52	-	-	-	-	-	-	-	-
		Mongols	12	-	-	-	-	-	-	-	-

6.5 Reporter gene assays on *LCT* promoter variants

Functional studies were conducted to examine the two most interesting *LCT* promoter variants. The variants -17^*T and -36^*T were chosen for analysis because, as described above, -17^*T was found in many African and some other populations and -36^*T was more common in pastoralists groups from Africa (Jones 2012). Their locations in a region of binding for several transcription factors (Figure 6.2) also make them interesting for study. With the comparison of the promoter region with other primate species (Appendix A, or also seen in Figure 6.2) it was recognised that -36^*T is actually the ancestral variant for this SNP. However, -36^*C is the common variant in all populations so far and for comparison reasons with previous work (Jones 2012) I will further refer to -36^*T as the variant.

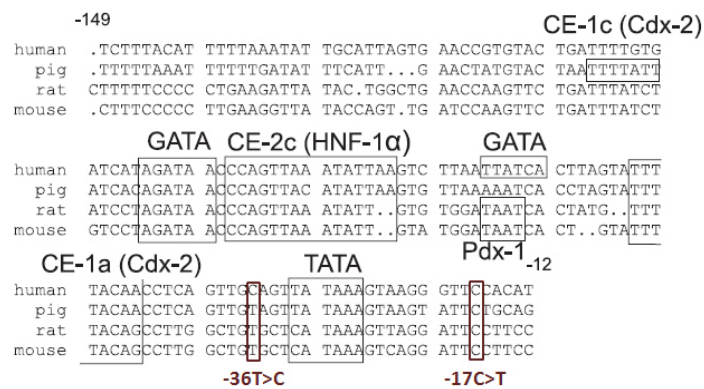


Figure 6.2: Alignment of 150 bp of the proximal *LCT* promoter showing the location of the two variants chosen for transfection experiments (red boxes). *Cis*-elements are indicated by black boxes and transcription factors binding are shown. Picture modified from Troelsen (2005).

The two *LCT* promoter variants were introduced into the reporter gene plasmids containing the ancestral enhancer sequence, upstream of the firefly luciferase gene (*luc+*) (for details see section 2.2.7.2) and transfected into Caco2 cells as described in chapter 2 (section 2.2.7). Gel shift assays were not conducted since these are inappropriate for promoter regions where multiple proteins are binding.

Transfection studies revealed no significant difference in reporter gene expression between the control plasmids and the constructs containing the -36^*T and -17^*T . Figure 6.3 shows the relative luciferase activity after 2 and 9 days of transfection compared to the ancestral promoter only plasmid and also that containing the ancestral enhancer sequence, with promoter enhancer construct carrying -17^*C and -36^*C .

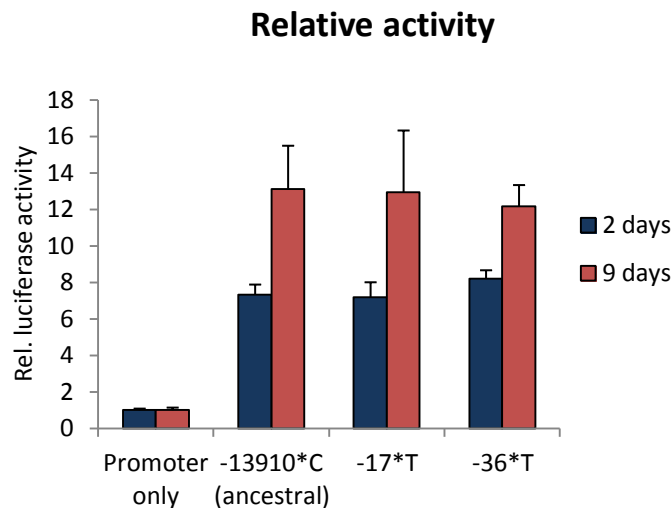


Figure 6.3: Result of luciferase reporter gene assay for the *LCT* promoter variants in constructs including the ancestral enhancer sequence, after 2 and 9 days of transfecting the Caco-2 cells. Luciferase activity of promoter variant constructs are shown in comparison with the promoter only construct. Luciferase activities (means \pm SD, n=4) were corrected for transfection efficiency and normalised to the expression of pGL3 hLPH1085 (promoter only). Neither variant allele showed significantly different expression from the ancestral allele.

6.6 Variation in intron 2 of *LCT*

Data for *LCT* intron 2 were collected for a selection of European and Middle Eastern populations from chapter 1 and phenotyped samples from Europe, Asia and Africa.

The *LCT* intron 2 region was successfully sequenced for 308 samples and Table 6.2 shows the allele frequencies in 11 populations tested. 13 variants were detected, of which 5372 C>T, 5473 C>T and a 4 bp deletion, around position 5788 from the start of transcription of *LCT*, were present in all populations studied.

To explore a possible association with the lactase persistence phenotype previously phenotyped populations (see also chapter 2) from Northern Europe (Harvey et al. 1995a) and Italy (Harvey et al. 1998), the Siberian Yakuts (Hollox 2000) and the African Jaali and Somali (Ingram et al. 2007; Ingram et al. 2009b) and an additional set of 26 phenotyped Finnish individuals (Harvey et al. 1998; Hollox 2000) were further analysed. After exclusion of samples with missing data at more than 4 positions in the *LCT* intron 2 or enhancer sequence regions 172 samples were examined.

Allele counts for the entire group and for the group divided into European + Asian and African samples are shown in Table 6.3. For simplicity lactase persistent samples as tested

by biopsy and lactose digesters tested by breath hydrogen tests were both grouped as lactase persistent (and vice versa for non-persistent individuals). Phenotype counts were then used to build a 2x2 table to examine association with Fishers exact test.

The two alleles *5473*T* and *5586*A* show a significant association with lactase persistence in the overall sample set, whereas *5372*T* and the 4 bp deletion at position 5788 are instead associated with lactase non-persistence. Since it seemed to be very likely that those associations reflect demographic patterns, samples were split according to their origin into a European/Asian and African sample set.

*5473*T* was found in both, the European/Asian and African sample sets and is not significant associated with persistence when both sets were analysed separately (Table 6.3). As shown in Table 6.2, *5586*A* was only found in Europeans and 18/21 of the chromosomes were in persistent people (Table 6.3). This suggested that it might be associated with the *-13910*T* carrying chromosomes. However, the three *5586*A* variants occurred in non-persistent individuals who do not carry the **T* allele but all of whom were previously shown by former members of the lab to carry at least one A haplotype which would support the suggestion that *5586*A* occurs on an A haplotype background and preliminary PHASE analysis points to this.

The 4 bp deletion at position 5788 remained significantly associated with lactase non-persistence in the African sample subset. This variant was mainly found on a B haplotype background (*-958*T*, *666*A*) but was also found on the core A, C and other haplotypes as assessed by visual inspection of the data and preliminary PHASE analysis. This result would need further confirmation but suggests that some gene conversion has occurred.

Critically, no alleles were found exclusively in persistent people, including those with no functional enhancer alleles.

Table 6.2: Allele frequencies of *LCT* all intron 2 variants observed in a subset of European and Middle Eastern populations from chapter 3 and additional phenotyped samples, N: Number of chromosomes.

		rs11886852	rs9636213		rs10928551		rs10928550							
Population	N	5372 C>T	5409 C>T	5473 C>T	5586 G>A	5623 G>A	5669 C>A	5698 C>T	5742 G>A	5783 G>C	5788 4 bp Indel (1=del)	5807 C>T	5810 C>T	6013 C>T
Norwegians	52	0.058	-	0.942	0.019	0.019	-	-	-	-	0.058	-	-	-
Greek-Cypriots	56	0.446	-	0.536	0.036	-	-	-	0.107	-	0.464	-	-	-
Kuwaiti	44	0.341	-	0.591	-	-	0.023	-	0.068	0.023	0.341	-	-	0.023
Syrians	32	0.406	-	0.594	0.063	-	-	-	0.031	-	0.375	-	-	-
Yemeni Hadramaut	60	0.350	0.017	0.567	0.017	-	0.017	0.033	0.033	0.017	0.317	-	-	0.050
Other_ME	12	0.250	-	0.750	-	-	-	-	0.167	-	0.250	-	-	-
Phenotyped samples														
Northern Europeans	40	0.100	-	0.900	0.425	-	-	-	-	-	0.100	-	-	-
Italians	32	0.281	-	0.719	0.031	-	-	-	0.063	-	0.313	-	-	-
Yakuts	56	0.143	-	0.821	-	-	-	-	-	-	0.179	0.018	0.018	-
Jaali	110	0.382	-	0.564	-	-	0.082	0.009	0.036	0.091	0.273	-	-	0.018
Somali	122	0.467	-	0.352	-	-	0.098	0.008	-	0.057	0.484	-	-	0.008

Table 6.3: Allele counts and association of the *LCT* intron 2 variants. Statistically significant *p*-values after Bonferroni correction for 11 tests (0.0045) are shaded. P: lactase persistent (or lactose digesters), NP: lactase non-persistent (or lactose non-digesters), N: Number of chromosomes.

Group		N	5372 C>T	5473 C>T	5586 G>A	5669 C>A	5698 C>T	5742 G>A	5783 G>C	5788 4 bp Indel	5807 C>T	5810 C>T	6013 C>T
All	P	162	35	115	18	6	0	2	6	26	1	1	0
	NP	182	73	94	3	9	1	3	6	72	0	0	1
<i>p</i> -value			<0.001	<0.001	<0.001	0.609	1	1	1	<0.001	0.471	0.471	1
Europeans/ Asians	P	82	10	71	18	0	0	1	0	11	1	1	0
	NP	90	24	62	3	0	0	2	0	26	0	0	0
<i>p</i> -value			0.021	0.015	<0.001	-	-	1	-	0.015	0.482	0.482	-
Africans	P	80	25	44	0	6	0	1	6	15	0	0	0
	NP	94	49	32	0	9	1	1	6	46	0	0	1
<i>p</i> -value			0.006	0.006	-	0.788	1	1	0.774	<0.001	-	-	1

6.7 Discussion

During this study the *LCT* promoter near to the start of transcription was surveyed in many European and Middle Eastern samples. As previously suggested for Europeans, this region is quite conserved and only a few variants were found. This same region was also studied in a parallel project in Africans (Jones 2012). Of the frequent African variants, -173*A and -17*T were also present in the sample set of this study, mainly in the Middle East, although at low frequencies.

However, the evidence from the African groups of a possible connection between a pastoralist lifestyle and the occurrence of -36*T and -17*T motivated the inclusion of these two variants in functional studies. Transfection studies were conducted to test the effect of the varying promoter sequences at these positions. No influence on reporter gene expression could be detected. This negative finding however agrees with the further developments of the parallel African studies, namely association studies with lactose digester status in Ethiopians, which failed to show any evidence of association with persistence making a functional role unlikely (Jones 2012).

In a search for possible new regions with variation that could be causal for lactase persistence, results of ChIP-Seq analysis conducted by our colleagues in the laboratory in Copenhagen (Boyd et al. 2009; Boyd et al. 2010) were used to identify a region in intron 2 of *LCT* which had been shown to be target for Cdx-2 and HNF-4 α binding.

In contrast to the promoter, the other region of interest, intron 2 of *LCT*, revealed relatively more variations. Some of the alleles were rather frequent and present in both persistent and non-persistent people discounting a direct functional role. Some were more frequent in lactase non-persistent individuals and others were more frequent in the persistent individuals.

The only possible candidate SNP for function is 5586 G>A since only three individuals classed as non-persistent carried the allele. However all 18 derived alleles that occurred in persistent individuals also carried at least one -13910*T allele. Clearly not all -13910*T allele carrying persistent individuals carry 5586*A – since the allele was at low frequency in some places where the *T allele is common, such as Norway. This suggests that it is a derived allele on the background of A, -13910*T chromosomes, and thus non-essential for lactase persistence. Likewise the three individuals who carry this allele and not -13910*T are non-persistent showing that it cannot on its own cause persistence, and indicating some recombination/gene conversion has occurred.

Nevertheless, it can be speculated that binding of HNF-4 α and Cdx-2 may differ for the ancestral and derived allele at this position and this might interplay with the Oct-1 binding of the -13910 position and the transcription factors binding to the promoter to modulate *LCT* transcription, possibly causing inter-individual differences in lactase expression and persistence. Further functional studies could be conducted to test this *in vitro*, to search for evidence of that SNP altering the binding of specific transcription factors. The case for doing that is however not supported by the bioinformatic examination of the effect of the SNP on the probability of the predicted Cdx-2 binding which did not differ between the derived and ancestral allele.

In summary, no new highly putative causal alleles were found. Disappointingly it was not possible to include the Wolof in these DNA analyses since the quality of the remaining DNA samples in the lab was poor. The variants in intron 2 appear to disrupt the haplotypes described in Chapter 5 in a way that was unexpected, and suggesting gene conversion events and this might have merited further examination, but the datasets were not completely overlapping and further testing was not warranted by the lack of good candidates in this region.

7 General Discussion

One of the main aims of this thesis was to examine the genetic regions in and around *LCT* for variation and collect data about known lactase persistence associated alleles in geographic regions where there was less dense information available, mainly Eastern European and West Asian populations. The vast amount of enhancer variation data assembled in chapter 3 should significantly contribute to the knowledge about the distribution of *LCT* enhancer alleles. The Middle East, as contact zone between Europe, Africa and Asia revealed variation patterns reflecting influences from all these continents in both, the enhancer and the *LCT* surrounding markers as seen in the haplotype variation.

In chapter 4 it could be shown that two further *LCT* enhancer alleles alter function *in vitro*. This increases the number of functional variants likely to be responsible for the lactase persistence trait to six: -14011*T, -14010*C, -14009*G, -13915*G, -13910*T and -13907*G. Our recently published study (Jones et al. 2013) revealed the association of -14009*G with lactase persistence in a 350 individuals Ethiopian cohort and confirmed the association of -13915*G and -13907*G.

The functional studies described here show that -14009*G and also -14011*T have a clear effect on transcription factor binding *in vitro*. That in the case of -14009*G is quite different from -14010*C and -14011*T, showing that even when a small DNA binding region is involved more than one different route can lead to alteration of function. -14011*T is not frequent enough to test for association. It was found scattered as a rare variant in several populations in Europe and the Middle East in this thesis and in further studies in Estonia, Ethiopia and even in indigenous populations from Brazil (Friedrich et al. 2012; Jones et al. 2013; Lember et al. 2006). This together with the finding of the haplotype analyses of chapter 5, that showed both A and B haplotypes as background, leads to the suggestion that this allele might have arisen by mutation independently several times. The lack of evidence of a single extended haplotype is slightly surprising and is not consistent with recent selection. A further allele -14028*C is almost certainly functional as well since it was associated with high lactase RNA expression (Poulter et al. 2003).

Further downstream, variants -13806*G and -13730*G were shown not be lactase persistence associated (Jones et al. 2013) although located in the same region with enhancer function (Troelsen et al. 2003). These and other new variants found in the

investigations reported in chapter 3 and 5 are single variants or not frequent enough to be worth examining their influence on *LCT* expression without other reason.

One of the relatively strong candidate functional alleles -13779*C, frequent in Indians tested in this thesis and elsewhere (Gallego Romero et al. 2012) showed no difference in protein binding in the EMSA studies and no significant difference in reporter gene activity in the experiments conducted during this thesis. From this evidence it can be concluded that it does not alter function. However, the -13779 position or the surrounding 12 bp sequence contains a binding site for HNF-4 α which might not have direct influence on the lactase persistence trait but is possibly part of the transcription factor network involved in regulation.

It is striking that the functional variants cluster in two regions indicating that these are the 'functional hotspots' of the enhancer region. However this does not *per se* mean that mutations there always influence function, as shown for -13913*C (Enattah et al. 2008; Ingram et al. 2007) which is not associated with lactase persistence (Jones et al., 2013). This is another example of how very closely located mutations can have a different effect of transcription factor binding.

The complex interactions of different transcription factors binding to the enhancer and promoter of the *LCT* gene were reported previously (reviewed in Montgomery et al. 2007) and further experiments would be needed to investigate the role of the two SNPs -14011 C>T and 14009 T>G in this network of *cis* and *trans* regulatory elements. This could be done for example in further reporter gene essays under the co-transfection of other transcription factor proteins, known to be involved in intestinal regulation.

One of the important findings of chapter 4 was the binding of an additional protein to -14009*G, which is suggested to be an Ets factor, as evidenced from EMSA experiments with tailored competitor oligonucleotide sequences. It is quite likely that a factor of this protein family is involved *in vivo* as they play a role in cell differentiation and maintenance (Sharrocks 2001). EMSA experiments with further antibodies against c-Ets-1, ELK-1 or other Ets factors might be able to confirm an interaction with the variant at -14009. Another way of identifying the protein would of course be its isolation from gels, purification and protein sequencing, or with the fractionation of the Caco2 nuclear extract as done by Lewinsky and colleagues for the -13910*T binding Oct-1 and GAPDH proteins in Caco2 and HeLa cells (Lewinsky et al. 2005). However, this requires an enormous

amount of time and material and the antibody approach might therefore be the first choice.

Of course *in vitro* studies do have their limitations, as conditions in the living organism differ and the complex biochemical settings in a cell of a living organism might differ from the *in vitro* situation, though ideally, the effects of variants are better studied *in vivo* as recently done for -13910*T. A 218 bp enhancer fragment carrying the variant, fused to a 2 kb rat promoter was sufficient to prevent the down-regulations of lactase in mice (Fang et al. 2012).

However, it is difficult and costly to establish transgenic mouse lines and many aspects of *LCT* regulation are different in the mouse. It might be more appropriate to study lactase persistence directly in measuring mRNA levels and chromatin alterations in the human proximal jejunum. In projects that collaborate with hospitals outside Europe and collect intestinal biopsy material for other reasons, the association of allelic expression levels with other enhancer variants could be examined, together with chromatin technologies to examine TF/chromatin complexes in the context of development. This way changes in transcription factor binding as it occurs *in vivo* can be confirmed.

Another point was recognised during the experimental phase for chapter 4. As already mentioned in the discussion of that chapter, the enhancer variants tested in all the transfection studies in the Danish lab were tested in a construct with -958*T in the promoter sequence, which does not reflect the *in vivo* situation for variants other than -14028*C.

To exclude possible confounding influence of -958*T and other variable promoter positions, experiments should be repeated with a promoter carrying -958*C. This would be especially important as the T* allele at that position was shown in previous EMSA experiments to disrupt binding of a protein (Hollox et al. 1999), which is probably Oct-1 as suggested from experiments of this thesis, and this variant also decreases reporter gene expression (Chitkara et al. 2001). For the -13915 T>C variant mutagenesis of the -678*A to -678*G should also be considered to mirror the haplotype background of this allele. A further mutation of the construct for -14009*G at the InDel position -943/942 is probably less important as this was shown to not affect protein binding (Hollox et al. 1999).

However, functional studies of both kinds might also lack genetic regions in the region of *LCT* that could have an influence on expression. The updated GenoPheno correlation map of chapter 3 revealed that there are still geographic regions where there is a difference

between the lactase persistence genotype and phenotype frequency, which implies the possible existence of other functional elements responsible for lactase persistence.

Scanning the genome for transcription factor binding sites is possible with immunoprecipitation methods, which can also identify other regulatory influences, such as DNA methylation and the binding of RNA polymerase II, which could be helpful in identifying possible new target regions (reviewed in Mikkelsen et al. 2007; Olsen et al. 2012). Data for proteins with a restricted expression in the intestine and therefore relevant to lactase can be collected from Caco2 cells as it has been done with HNF-4 and Cdx-2 by Boyd and colleagues (2009; 2010). Preliminary experiments failed so far to establish a similar assay for Oct-1 (Troelsen, personal communication).

The next generation sequencing technologies allow large scale genome scans for elements regulating gene expression, also including the detection of small interfering RNA molecules and allelic transcript expression by RNA sequencing (RNA-Seq, for example Chia et al. 2010). Further techniques can scan for open chromatin structures such as DNase-Seq (DNase I hypersensitivity sequencing) or FAIRE (Formaldehyde-Assisted Isolation of Regulatory elements)-Seq (Giresi et al. 2007), which was recently modified to work with gene chips (FAIRE-gen) to be suitable for GWAS (Smith et al. 2012). Projects like ENCODE (Bernstein et al. 2012, <https://genome.ucsc.edu/encode/>) have the goal to combine and annotate data collected with these techniques and accumulate knowledge about regulatory elements and gene expression.

Research in the past focussed on protein structure or exon sequencing to identify genetic causes for monogenic or polygenic traits. That this still is case shows the data of one of the most popular research projects, the 1000 genomes project. It provides high coverage data for exonic regions (at least 20 x) but only low (1-2 times on average) for non-exonic regions (Abecasis et al. 2010, <http://www.1000genomes.org/>), though the coverage is higher for the first 100 -200 bp of non-coding regions. It was for example not possible to use 1000 genomes data for this thesis as the enhancer region was only covered 2x on average for each individual which of course cannot reliably distinguish heterozygotes, even an expansion of the data to a data coverage of 4-8x (Tyler-Smith, personal communication 2012) would not have been reliable enough.

The haplotype analyses in chapter 5 gave further insights into the occurrence and constitution of the haplotypes of the region surrounding *LCT*. Remarkable was the

frequent occurrence of a frequent B haplotype, which was of similar length to those haplotypes carrying functional variants and suggested to be the result of selection events. Rather surprising was the paucity of derived functional variants on the B haplotype. This haplotype forms the background of only two relatively rare derived enhancer alleles: -13913*C, which does not seem to cause LP, and -14028*C. The latter has been found in two European individuals so far (in one sample examined in this thesis) and was shown to reside on a high lactase expressing chromosome (Poulter et al. 2003). It was confirmed that the -14028 position is part of a Cdx-2 binding site as previously shown (Lewinsky et al. 2005). The derived allele leads to a visible loss of binding to that transcription factor in the EMSA studies conducted but also gains binding to another transcription factor shown to be highly involved in lactase expression, HNF-4 α . However, a functional effect could not be shown with the transfection studies conducted in this thesis (chapter 4). It should also be noted that the British person carrying -14028*C was only 21 years of age, which could mean that the decline of lactase expression had not fully happened, as shown for some Finnish individuals at the same age in a different study (Sahi et al. 1983). In this context -14028*C may have a function by simply delaying this process.

In chapter 5, the results from EHH analysis showed long stretches of homozygosity in the same regions where the linkage disequilibrium maps showed clear evidence of suppression of recombination, which exists in populations with and without lactase persistence. This leads to the question to what degree methods based on extended haplotype homozygosity to detect recent selection events are influenced by historical recombination patterns reflected for example in recombination hot or cold spots of the genome. It is of noteworthy that *LCT* is surrounded by genes that are important for cell function, translation and replication stress response, which might possibly favour reduced diversity.

Allele specific suppression of recombination in the region around *LCT* has previously been discussed as a possible factor that might create a similar pattern of an extended haplotype to that of a recent selection event (Hollox 2005), in the context of the -13910*T allele. A different strategy involving examination of microsatellite diversity around *LCT* did in that case however support the evidence for selection (Coelho et al. 2005).

Although *MCM6* and *LCT* are expressed at different developmental stages of an intestinal cell, (*MCM6* is highest in foetuses and high in intestinal crypts but down regulated when lactase activity is highest in the differentiated state), it cannot be excluded that they do not influence each other. It was suggested that *MCM6* expression in less differentiated cells has

an impact as precursor on chromatin packaging that also allows easier accessibility of transcription factors to the *LCT* promoter (Troelsen 2005). Maybe other neighbouring genes have an influence on the chromatin state of that region.

LDU maps take current and historical recombination patterns into account when comparing linkage disequilibrium between alleles in populations. Regions of similar LDU therefore reflect population specific evolutionary influences such as mutation, drift and most importantly, selection. It would therefore be desirable to examine the populations for the markers used for extended haplotype analyses in chapter 5 with both the LDU approach and EHH tests in each of the populations to be able to compare both methods and get quantitative calculable evidence for selection (Tapper 2007). The SNP data generated here were unfortunately of much lower density than that used for the LDU maps.

The main questions that remain unanswered and could be the motivation for future work are: Is there something about the B haplotype that impedes adult *LCT* expression when mutations do occur? Does the long region of LD included within the B haplotype give us any clues? What is suppressing recombination in this region and could this be allele specific? Can we exclude that there is not a large inversion within this long sequence region, perhaps on B haplotype chromosomes?

However, the occurrence of the lactase persistence trait in various parts of the world, enabled by different recent variants in the enhancer element of the *LCT* gene and each on long haplotypes, is strongly supportive evidence, of selection triggered by the adaptation to a new diet. The question is how this new diet influenced the life of the early Neolithic farmers and how milk consumption was so beneficial.

The shift to agriculture was associated with a reduction in diversity of the nutritional intake but also an excess of caloric availability, mainly in form of starch (Luca et al. 2010). For early Neolithic farmers the transition from a hunter-gatherer lifestyle to farming was most probably accompanied by periods of crop failure and domesticated animals built an additional food supply during these times. Even when taking the lower productivity of early domesticated cows into account and subtracting the milk needed for raising calves, a remarkable surplus of milk would have been produced (Gerbault et al. 2011).

Even if evidence for milk use exists in early Neolithic societies from Anatolia and the Balkans (Craig et al. 2005; Evershed et al. 2008) it is unlikely that people were able to

consume large quantities of fresh milk, as ancient DNA data suggest (Figure 7.1). The earliest evidence for the lactase persistence associated allele was found in samples from Scandinavian late hunter-gatherers from about 5400-3400 BP with a frequency of 5% (Malmstrom et al. 2010) and in Spanish early Neolithic farmers, dated to about 5000-4500 BP with a T^* allele frequency of 11 and 26% (Plantinga et al. 2012). Data from Mesolithic and early Neolithic samples of other regions of Europe suggest the absence of the lactase persistence trait in these populations (Burger et al. 2007; Lacan et al. 2011a; Lacan et al. 2011b).

It is very likely that early farmers would have gained nutritional benefits from dairying in form of processed milk products such as yoghurt or cheese. This would have also been more feasible as these are more durable forms of milk which are easier to transport, probably important for early farmers that had rather seasonal settlements (reviewed in Leonardi et al. 2012). A recent study on pot shards from Poland dated to about 7400-6800 BP, which show the shape of sieves used for cheese production and contain ruminant fat residues, directly supports this argument (Salque et al. 2013). For the milk residues found earlier, 8500 BP in Anatolia and the Balkans from around 7900-7500 BP it was also suggested that they could only last this long time if fermented milk was used in these vessels (Craig et al. 2005).

The fermentation process would have reduced the amount of lactose, which would have also allowed non-persistent farmers to profit from the nutritional value of milk. Therefore Burger and Thomas (2011) suggest the rise of -13910^*T would have started after 5500 when dairying practises had been established and with a better supply of fresh milk the ability to drink it became more advantageous.

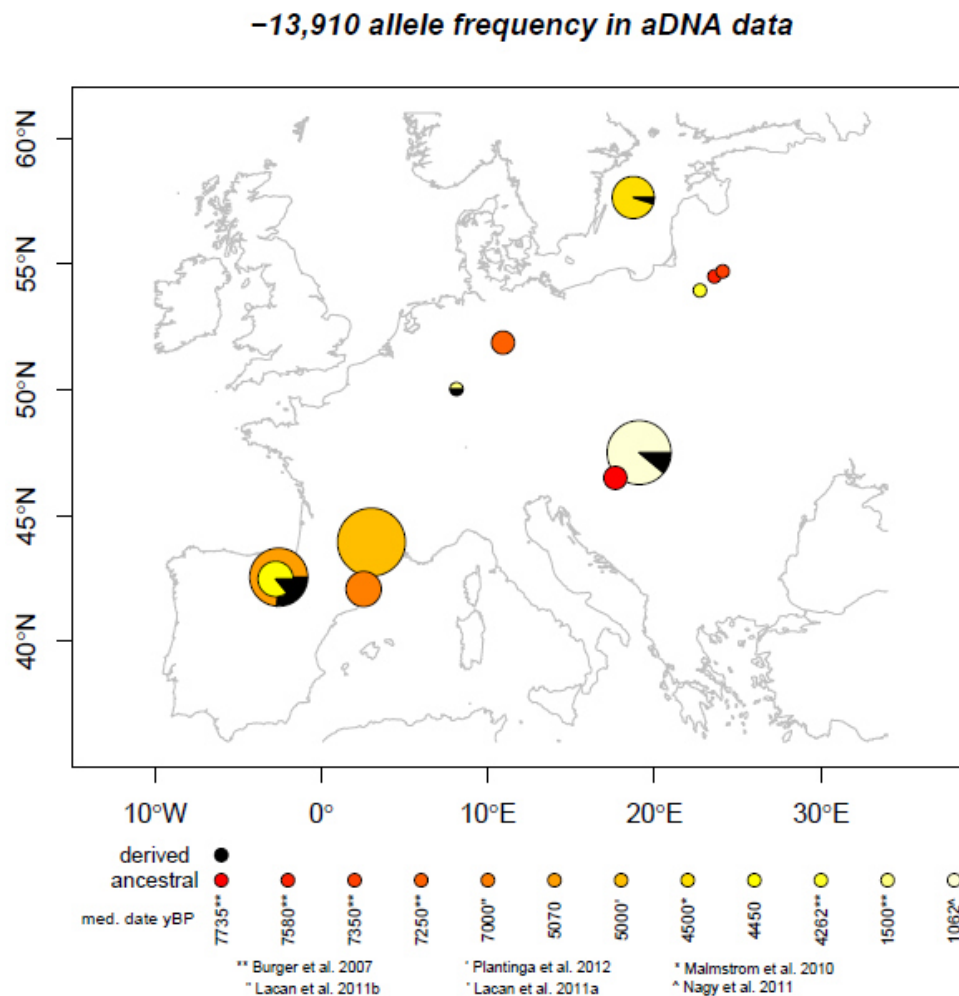


Figure 7.1: Frequency of -13910*T as revealed by ancient DNA analysis and location of the sample origins. Circle diameter correspond to sample sizes (map kindly provided by P. Gerbault).

The characteristic molecular signatures of purifying and positive selection are a decrease in diversity in the adjacent genetic regions of the selected mutation and an increase of this diversity for balancing selection and soft selective sweeps. Both are seen for the *LCT* enhancer. However, the ways selection operates to increase, decrease or maintain genetic diversity are complex i.e. allele frequencies can be variable and processes involved difficult to retrace through space and time. In general, genetic drift and demography affect the whole genome whereas selection tends to act on the selected genes or regions only. As mentioned earlier, it can be difficult to distinguish selection from demographic events and statistical tests can help to build up the evidence for a certain scenario. The combination of different tests and simulation models that compare simulated variables with actual data from various scientific disciplines such as archaeology and anthropology as described above (chapter 1, section 1.2.15) seems to be a good way forward.

The most recent study that builds upon that approach was done by Pascale Gerbault (2013) also using a coalescent simulation model paired with approximate Bayesian computation (ABC) to model the spread and origins of lactase persistence across Europe but taking further and weighted data into account. Statistics that fit best with data from archaeological records including dairy fat residues in potsherds, and ancient and modern DNA data (partly from this thesis) for *-13910*T* were evaluated. Exchanges between hunter-gatherers and farmers were allowed in form of cultural diffusion, gene flow and density-dependent competition between hunter-gatherers and farmers. In comparison to Itan et al. (2009) this model suggests a more recent time of about 5000 years to be the starting point of selection for lactase persistence, placing it in Northern rather than Eastern Europe (Gerbault 2013). This shows how sensitive this kind of study is to the correct selection of realistic parameters.

Nevertheless, with the further development of the simulation models it might also be possible in the future to take other *LCT* enhancer alleles into account and model the spread of lactase persistence outside of Europe, which might reveal further insights into the evolutionary past of this trait and provide answers in relation to the different sweeps involved.

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Appendices

Appendix A: Sequence alignments of the regions amplified during this thesis. Nucleotide positions in relation to the start of transcription of *LCT*. Positions of variants found are highlighted. Aligned species: Homo sapiens (H. sapiens), Pan troglodytes (P. troglod.), Gorilla gorilla (G. gorilla) and Pongo abelii (P. abelii).

LCT enhancer, intron 13 of MCM6

	-14390	-14380	-14370	-14360	-14350	-14340	-14330	-14320	-14310	-14300	-14290	-14280	-14270	-14260
H.sapiens	ATTTCCAAAGAGTCAGAGGACTTCATTGTGGAGCAATATAAACATCTCCGCCAGAGAGATGGTTCTGGAGTGACCAAGTCTTCATGGAGGATTACAGTGCGACAGCTTGAGAGCATGATTTCGTCTCTCTGAAGCTATGGC													
P.troglod.	ATTTCCAAAGAGTCAGAGGACTTCATTGTGGAGCAATATAAACATCTCCGCCAGAGAGATGGTTCTGGAGTGACCAAGTCTTCGTGGAGGATTACAGTGCGACAGCTTGAGAGCATGATTTCGTCTCTCTGAAGCTATGGC													
G.gorilla	ATTTCCAAAGAGTCAGAGGACTTCATTGTGGAACAATATAAACATCTCCGCCAGAGAGATGGTTCTGGAGTGACCAAGTCTTCGTGGAGGATTACAGTGCGACAGCTTGAGAGCATGATTTCGTCTCTCTGAAGCTATGGC													
P.abelii	ATTTCCAAAGAGTCAGAGGACTTCATTGTGGAACAATATAAACATCTCCGCCAGAGAGATGGTTCTGGAGTGACCAAGTCTTCGTGGAGGATTACAGTGCGACAGCTTGAGAGCATGATTTCGTCTCTCTGAAGCTATGGC													
	-14250	-14240	-14230	-14220	-14210	-14200	-14190	-14180	-14170	-14160	-14150	-14140	-14130	-14120
H.sapiens	TCGGATGCACTGCTGTGATGAGGTATCAGAGTCACCTTTGATATGATGAGAGCAGAGATAAACAGATTGTTGTCATGTTTTTAATCTTTGGTATGGGACATACTAGAATTCACCTGCAAAATACATTTTTATGTAACGTGTTGA													
P.troglod.	TCGGATGCACTGCTGTGATGAGGTATCAGAGTCACCTTTGATATGATGAGAGCAGAGATAAACAGATTGTTGTCATGTTTTTAATCTTTGGTATGGGACATACTAGAATTCACCTGCAAAATACATTTTTATGTAACGTGTTGA													
G.gorilla	TCGGATGCACTGCTGTGATGAGGTATCAGAGTCACCTTTGATATGATGAGAGCAGAGATAAACAGATTGTTGTCATGTTTTTAATCTTTGGTATGGGACATACTAGAATTCACCTGCAAAATACATTTTTATGTAACGTGTTGA													
P.abelii	TCGGATGCACTGCTGTGATGAGGTATCAGAGTCACCTTTGATATGATGAGAGCAGAGATAAACAGATTGTTGTCATGTTTTTAATCTTTGGTATGGGACATACTAGAATTCACCTGCAAAATATATTTTTATGTAACGTGTTGA													
	-14110	-14100	-14090	-14080	-14070	-14060	-14050	-14040	-14030	-14020	-14010	-14000	-13990	-13980
H.sapiens	ATGCTCATACGACCATGGAATTCTTCCCTTTAAAAAGCTTGGTAAAGCATTGAGTGTAGTTGTTAGACGGAGACGATCACGTCATAGTTTATAGAGTGCATAAAAGACGTAAGTTACCATTTTAATACCTTTTCATTACAGGAA													
P.troglod.	ATGCTCATACGACCATGGAATTCTTCCCTTTAAAAAGCTTGGTAAAGCATTGAGTGTAGTTGTTAGACGGAGACGATCACGTCATAGTTTATAGAGTGCATAAAAGACGTAAGTTACCATTTTAATACCTTTTCATTACAGGAA													
G.gorilla	GTGCTCATATGACCATGGAATTCTTCCCTTTAAAAAGCTTGGTAAAGCATTGAGTGTAGTTGTTAGACGGAGACGATCACGTCATAGTTTATAGAGTGCATAAAAGACGTAAGTTACCATTTTAATACCTTTTCATTACAGGAA													
P.abelii	GTGCTCATAGGACCATGGAATTCTTCCCTTTAAAAAGCTTGGTAAAGCATTGAGTGTAGTTGTTAGACGGAGATGATCACGTCATAGTTTATAGAGTGCATAAAAGACGTAAGTTACCATTTTAATACCTTTTCATTACAGGAA													
	-13970	-13960	-13950	-13940	-13930	-13920	-13910	-13900	-13890	-13880	-13870	-13860	-13850	-13840
H.sapiens	AAATGTACTTAGACCTTACCAATGTACTAGTAGGCCCTCTGCGCTGGCAATACAGATAAGATAATGTAGTGGCCCTGGCCTCAAAGGAACCTCTCCTCCTTAGGTTGCATTTGTATAATGTTTGATTTTATAGATTGTTCTTTGAG													
P.troglod.	AAATGTACTTAGACCTTACCAATGTACTAGTAGGCCCTCTGCGCTGGCAATACAGATAAGATAATGTAGTGGCCCTGGCCTCAAAGGAACCTCTCCTCCTTAGGTTGCATTTGTATAATGTTTGATTTTATAGATTGTTCTTTGAG													
G.gorilla	AAATGTACTTAGACCTTACCAATGTACTAGTAGGCCCTCTGCGCTGGCAATACAGATAAGATAATGTAGTGGCCCTGGCCTCAAAGGAACCTCTCCTCCTTAGGTTGCATTTGTATAATGTTTGATTTTATAGATTGTTCTTTGAG													
P.abelii	AAATGTACTTAGACCTTACCAATATATACTAGTAGGCCCTCTGCGCTGGCAATACAGATAAGATAATGTAGTGGCCCTGGCCTCAAAGGAACCTTTGGTCTCTTAGGTTGCATTTGTATAATGTTTGATTTTATAGATTGTTCTTTGAG													
	-13830	-13820	-13810	-13800	-13790	-13780	-13770	-13760	-13750	-13740	-13730	-13720	-13710	-13700
H.sapiens	CCCTGCATTCCACGAGGATAGGTCACTGGGTATTAAAGAGGTAAAAAGGGGAGTAGTACGAAAGGGCAATTCAGCGTCCCATCTTCTCTTCAACGAAAGCAGCCCTGGCTTTTCTAGTTTATTAATAGGTTTGATGTAA													
P.troglod.	CCCTGCATTCCACGAGGATAGGTCACTGGGTATTAAAGAGGTAAAAAGGGGAGTAGTACGAAAGGGCAATTCAGCGTCCCATCTTCTCTTCAACGAAAGCAGCCCTGGCTTTTCTAGTTTATTAATAGGTTTGATGTAA													
G.gorilla	CCCTGCATTCCACGAGGATAGGTCACTGGGTATTAAAGAGGTAAAAAGGGGAGTAGTACGAAAGGGCAATTCAGCGTCCCATCTTCTCTTCAACGAAAGCAGCCCTGGCTTTTCTAGTTTATTAATAGGTTTGACGTGA													
P.abelii	CCCTGCATTCCACGAGGATAGGTCACTGGGTATTAAAGAGGTAAAAAGGGGAGTAGTACGAAAGGGCAATTCAGCGTCCCATCTTCTCTTCAACGAAAGCAGCCCTGGCTTTTCTAGTTTATTAATAGGTTTGATGTGA													
	-13690	-13680	-13670	-13660	-13650	-13640	-13630	-13620	-13610	-13600	-13590	-13580	-13570	-13560
H.sapiens	GGTCTCTCTTTGAAAAGGGGGTTTGGCTTTTTTTTACAGTGTGACTGAGGTATAATTTATAAAAAGGGAAATGTATGGCATGGTGAGTTTTTTCATACATACCTCTGTGAATACCCAGCTCAAGATCCAAAAACATTTCCAT													
P.troglod.	GGTCTCTCTTTGAAAAGGGGGTTTGGCTTTTTTTTACAGTGTGACTGAGGTATAATTTATAAAAAGGGAAATGTATGGCATGGTGAGTTTTTTCATACATACCTCTGTGAATACCCAGCTCAAGATCCAAAAACATTTCCAT													
G.gorilla	GGTCTCTCTTTGAAAAGGGGGTTTGGCTTTTTTTTACAGTGTGACTGAGGTATAATTTATAAAAAGGGAAATGTATGGCATGGTGAGTTTTTTCATACATCCTTGTGAATACCCAGCTTAAAGATCCAAAAACATTTCCAT													
P.abelii	GGTTCTCTTTGAAAAGGGGATTTGGCTTTTTTTTACAGTATGACTGAGGTATAATTTATAAAAAGGGAAATGTATGGCATGGTGAGTTTTTTCATACATCCTCTGTGAATACCCAGCTCAAGATCCAAAAACATTTCCAT													
	-13550	-13540	-13530	-13520	-13510	-13500	-13490	-13480	-13470	-13460				
H.sapiens	AATTTTCAGAAAGTTCCAAACCCCTGCCTCTTTTCAGTCTTAGCCCTCTTCCCTGAAAGTAACACTGTCTCCGACTTCAATCACTACTTTTTATCCACAGG													
P.troglod.	AATTTTCAGAAAGTTCCAAACCCCTGCCTCTTTTCAGTCTTAGCCCTCTTCCCTGAAAGTAACACTGTCTCCGACTTCAATCACTACTTTTTATCCACAGG													
G.gorilla	AATTTTCAGAAAGTTCCAAACCCCTGCCTCTTTTCAGTCTTAGCCCTCTTCCCTGAAAGTAACACTGTCTCTGACTTCAATCACTACTTTTTATCCACAGG													
P.abelii	AATTTTCAGAAAGTTCCAAACCCCTGCCTTTTTTCAGTCTTAGCCCTCTTCCCTGAAAGTAACACTGTCTCTGACTTCAATCACTACTTTTTATCCACAGG													

Intron 4, *MCM6*

	-30460	-30450	-30440	-30430	-30420	-30410	-30400	-30390	-30380	-30370	-30360	-30350	-30340	-30330
H.sapiens	ACCCTCAGATTTTCAGCAGGACTTAATTTTATGGGACATACAAAATGGAAGAAAAAGCTGAAGCAGCTCAAGTATGGAATAATTATTATCAGATGAATTTAATATTATTATATTGCTCTGATAATCATTCTCCTTATTTG													
P.troglod.	ACCCTCAGATTTTCAGCAGGACTTAATTTTATGGGACATACAAAATGGAAGAAAAAGCTGAAGCAGCTCAAGTATGGAATAATTATTATCAGATGAATTTAATATTATTATATTGCTCTGATAATCATTCTCCTTATTTG													
G.gorilla	ACCCTCAAATTTCAACAGGACTTAATTTTATGGGACATACAAAAGGAAAAGAAAAGCTGAAGCAGCTCAAGTATGGAATAATTATTATCAGATGAATTTAATATTATTATATTGCTCTGATAATCATTCTCCTTATTTG													
P.abelli	ACCCTCAGATTTTCAGCAGGACTTAATTTTATGGGACATACAAAAGGAAAAGAAAAGTTGAAGCAGCTCAAGTATGGAATAATTATTATCAGATGAATTTAATATTATTATATTGCTCTGATAATCATTCTCCTTATTTA													
	-30320	-30310	-30300	-30290	-30280	-30270	-30260	-30250	-30240	-30230	-30220	-30210	-30200	-30190
H.sapiens	GCTTTCTTACATTAACCTGGTAGCTTATAAGATGCACATATTTTACCAACAGCTTTGGGTAGGAGATGCTTATCTTATAAAGTATGAATCTAGATTGGGCTTCTAGTTTGGGGGGGGAACACAACTGTATAAAGGAACT													
P.troglod.	GCTTTCTTACATTAACCTGGTAGCTTATAAGATGCACATATTTTACCAACAGCTTTGGGTAGGAGATGCTTATCTTATAAAGTATGAATCTAGATTGGGCTTCTAGTTTGGGGGGGGAACACAACTGTATAAAGGAACT													
G.gorilla	GCTTTCTTACATTAACCTGGTAGCTTATAAGATGCACATATTTTACCAACAGCTTTGGGTAGGAG-----ATCTTATAAAGTATGAATCTAGATTGGGCTTCTAGTCTCTGGGGGGGGAACACAACTGTATAAAGGAACT													
P.abelli	GCTTTGTTACATTAACCTGGTAGCTTATAAGATGCACGATTTCTACCAACAGCTTTGGGTAGGAGATGCCTTATCTTATAAAGTGTGAGCTAGATTGGGCTTCTAGTTTGGGGGGAACACAACTGTATAAAGGAACT													
	-30180	-30172	-30164	-30154	-30144	-30134	-30124	-30114	-30105	-30095	-30085	-30077	-30067	-30057
H.sapiens	TCTTGACAA----GACATTTGAAGATGGACTAGATAATAGAAGATATGTAATTTTATGAAATTAATATTGTTTAA-TTATCTTGGTTGTGATAAATGATATTGTAACAG--GATTGCTTTTATTCTTAGAGATGAATGCT													
P.troglod.	TCTTGACAA----GACATTTGAAGATGGACTAGATAATAGAAGATATGTAATTTTATGAAATTAATATTGTTTAA-TTATCTTGGTTGTGATAAATGATATTGTAACAG--GATTGCTTTTATTCTTAGAGATGAATGCT													
G.gorilla	TCTTGACAACTCAAGACATTTGAAGATGGACTAGATAATAGAAGATATGTAATTTTATGAAATTAATATTGTTTAA-TTATCTTGGTTGTGATAAATGATATTGTAACAGAGGATTGCTTTTATTCTTAGAGATGAATGCT													
P.abelli	TCTTGACAA----GACATTTGAAGATGAGACTAGATAATAGAATATATGTAATTTTATGAAATTAATATTGTTTAAATATCTTGGTTGTGATAAATGATATTGTAACAGAAAGATTGCTTTTATTCTTAGAGATGAATGCT													
	-30047	-30037	-30028	-30018	-30008	-29999	-29989	-29985	-29975	-29965	-29955	-29946	-29936	-29926
H.sapiens	AAAAATTTTAGG-TATGAAGTTTCTTGATGTTTCACTGTGTT-CAAAATACATAGTAAAAA-----AACATGTATCTGTAATAATGTTAGCAGTTGTGTTGAA-GTGGAGGGTATAAAAAGGTGATTTTGAATTTTC													
P.troglod.	AAAAATTTTAGG-TATGAAGTTTCTTGATGTTTCACTGTGTT-CAAAATACATAGTAAAAA-----AACATGTATCTGTAATAATGTTAGCAGTTGTGTTGAA-GTGGAGGGTATAAAAAGGTGATTTTGAATTTTC													
G.gorilla	AAAAATTTTAGG-TATGAAGTTTCTTGATGTTTCACTGTGTT-CAAAATATATAGTAAAAAGGAAAAACATGTATCTGTAATAATGTTAGTAGTTGTGTTGAA-GTGGAGGGTATAAAAAGGTGATATGAAATTTTC													
P.abelli	AAAAATTTTAGGGTGTGAAGTTTCTTGATGTTTCACTGTGTTTCAAAATATATAGTAAAAAAGAAA-AAAACGTATCTATAAATAAT---AGCAGTTGTGTTGAA-GTGGAGGGTATAAAAAGGTGATTTTGAATTTTC													
	-29916	-29906	-29896	-29887	-29877	-29867	-29857	-29847	-29838	-29828	-29820	-29810	-29800	-29790
H.sapiens	TGTAATAATTATCTTAAAGCTTAGGAGAA-GATGGAGCATGGAACAGATATTTGGAGTCATTTTGAACAAAGAAAAAAA-TTATTTTTTAAATGA--GTAAAAAGTGAAGTTTTTTTTTCTCTGCTGCTTGAATCATCATGG													
P.troglod.	TGTAATAATTATCTTAAAGCTTAGGAGAA-GATGGAGCATGGAACAGATATTTGGAGTCATTTTGAACAAAGAAAAAAA-TTATTTTTTAAATGA--GTAAAAAGTGAAGTTTTTTTTTCTCTGCTGCTTGAATCATCATGG													
G.gorilla	TGTAATAATTATCTTAAAGCTTAGGAGAA-GATGGAGCATGGAACAGATAGTTGGAGTCATTTTGAACAAAGAAAAAAA-TTATTTTTTAAATGAAAGTAAAAAGTGAAGTTTTTTTTTCTCTGCTGCTTGAATCAGCATGG													
P.abelli	TGTAATAATT-----AAAGCTTAGGAGAAAGATGGAACATAGAACAGATATTTGGAGTCATTTTGAACAAAGAAAAAATTATTTTTTAAATGAGAGTAAAAAGTGAAGTTTTATTTT-CTCTGCTGCTTGAATCAGCATGA													
	-29780													
H.sapiens	AGT													
P.troglod.	AGT													
G.gorilla	AGT													
P.abelli	AGT													

Hapdef region

	-1160	-1150	-1140	-1130	-1120	-1110	-1100	-1090	-1080	-1070	-1060	-1050	-1040	-1030
H.sapiens	ATCCACATTCTACAGGTGACAAAAATAGAGGCACAAAGTTAAGTAATTTTGTTCAGGTGAGATTTAAACCCAGGCATTCTGACTCCTGTATAACCATTAAAGATATGCAGAGAAAAGAACTGGAAAAGATACATATTGCTGA													
P.troglod.	ATCCACATTCTACAGGTGACAAAAATAGAGGCACAAAGTTAAGTAATTTTGTTCAGGTGAGATTTAAACCCAGGCATTCTGACTCCTGTATAACCATTAAAGATATGCAGAGAAAAGAACTGGAAAAGATACATATTGCTGA													
G.gorilla	ATCCACATTCTACAGGTGACAAAAATAGAGGCACAAAGTTAAGTAATTTTGTTCAGGTGAGATTTAAACCCAGGCATTCTGACTCCTGTATAACCATTAAAGATATGCAGAGAAAAGAACTGGAAAAGATACATATTGCTGA													
P.abelii	ATCCACATTCTACAGGTGACAAAAATAGAGGCACAAAGTTAAGTAATTTTGTTCAGGTGAGATTTCAACCCAGGCATTCTGACTCCTGTAGAACCATTAAAGATACGCAGAGAAAAGAACTGGAAAAGATACATATTGCTGA													
	-1020	-1010	-1000	-990	-980	-970	-960	-950	-940	-930	-920	-910	-900	-890
H.sapiens	AGATACTTATTATAGGAAGAGGAGGGGGAGGGTGAAGGAATTTGCAAGTTTTTCATAGATGTTTCCATATTGTTTGAATCTGTTACAAAATATGTTTCAGCATATTTTAAAGAGAAAATTTGGGGCAAAAATACCTTATTT													
P.troglod.	AGATACTTATTATAGGAAGAGGAGAGGGGAGGGTGAAGGAATTTGCAAGTTTTTCATAGATGTTTCCATATTGTTTGAATCTGTTACAAAATATGTTTCAGCATATTTTAAAGAGAAAATTTGGGGCAAAAATACCTTATTT													
G.gorilla	AGATACTTATTATAGGAAGAGGAGGGGGAGGGTGAAGGAATTTGCAAGTTTTTCATAGATGTTTCCATATTGTTTGAATCTGTTACAAAATATGTTTCAGCATATTTTAAAGAGAAAATTTGGGGCAAAAATACCTTATTT													
P.abelii	AGATACTTATTCTAGGAAGAGGAGGGGGAGGGTGAAGGAATTTGCAAGTTTTTCATAGATGTTTCCATATTGTTTGAATCTGTTACAAAATATGTTTCAGCATATTTTAAAGA--AAATTTGGGGCAAAAGACTTATTT													
	-881	-873	-863	-853	-843	-833	-823	-813	-803	-793	-783	-773	-763	-753
H.sapiens	TTGT---ATTATGTAACAAATTTTAAAAATAATGTGTGGCTGGGTGCGCTGGCTCACACCTGTAATCCCAACACTTTAGGAGGCTGAGGCAAGAGGATTGCTTGAGCCAGGAGTTCAAGACCAGCCTGGGTGACATGGC													
P.troglod.	TTGT---ATTATGTAACAAATTTTAAAAATAATGTGTGGCTGGGTGCGCTGGCTCACACCTGTAATCCCAACACTTTAGGAGGCTGAGGCAAGAGGATTGCTTGAGCCAGGAGTTCAAGACCAGCCTGGGTGACATGGC													
G.gorilla	TTGT---ATTATGTAACAAATTTTAAAAATAATGTGTGGCTGGGTGCGCTGGCTCACACCTGTAATCCCAACACTTTAGGAGGCTGAGGCAAGAGGATTGCTTGAGCCAGGAGTTCAAGACCAGCCTGGGTGACATGGC													
P.abelii	TTGTTGTATTATGTAACAAATTTTAAAAATAATTTGTGTGGCTGGGTGCACTGGCTCACACCTGTAAGCCCAACACTTTAGGAGGCTGAGGCAAGAGGATTGCTTGAGCCAGGAGTTCAAGACCAGCCTGGGTGACATGGC													
	-743	-733	-723	-713	-703	-693	-683	-673	-663	-653	-643	-633	-623	-613
H.sapiens	AAAACTCCATCTCTACTAAAAATACAAAAAATTAGCCAGTCGTGGTGGCGCACACCTATGGTCCCACCTACCCAGGATGCTGAGATGGGAGGATCACTTGAGCCAGGAAGTCAAGGCTGCAGGAAGCTGTGATCGCACC													
P.troglod.	AAAACTCCATCTCTACTAAAAATACAAAAAATTAGCCAGTCGTGGTGGTGCACACCTATGGTCCCACCTACCCAGGATGCTGAGATGGGAGGATCACTTGAGCCAGGAAGTCAAGGCTGCAGGAAGCTGTGATCGCACC													
G.gorilla	AAAACTCCATCTCTACTAAAAATACAAAAAATTAGCCAGTCGTGGTGGCGCACACCTATGGTCCCACCTACCCAGGATGCTGAGATGGGAGGATCACTTGAGCCAGGAAGTCAAGGCTGCAGGAAGCTGTGATCGCACC													
P.abelii	AAAACTCCATCTCTACTAAAAATACAAAAAATTAGCCAGTTGTGGTGGCGCACACCTATGGTCCCACCTACCCAGGATGCTGAGATGGGAGGATCACTTGAGCCAGGAGTCAAGGCTGCAGGAAGCTGTGATCGCACC													
	-603	-593	-583	-573	-563	-553	-543	-533	-523	-513	-503	-493	-483	-473
H.sapiens	ACTGCACCTCCACCTGGGCAACAGAGTGAGACCCGGTCAACCAAAAAACAAAAAAACAAAAAAATTGGTAATCGTTTTCTTCAGACATTTTCCGGGTTCCCTCTGCTTAACCTTGATAGGAAGTCTGAGGTTTTTGTGTT													
P.troglod.	ACTGCACCTCCACCTGGGCAACAGAGTGAGACCCGTGTCAACCAAAAAACAAAAAAACAAAAAAATTGGTAATCGTTTTCTTCATACATTTTCCGGGTTCCCTCTGCTTAACCTTGATAGGAAGTCTGAGGTTTTTGTGTT													
G.gorilla	ACTGCACCTCCACCTGGGCAACAGAGTGAGACCCGGTCAACCAAAAAACAAAAAAACAAAAAAATTGGTAATCGTTTTCTTCAGACATTTTCCGGGTTCCCTCTGCTTAACCTTGATAGGAAGTCTGAGGTTTTTGTGTT													
P.abelii	ACTGCACCTCTACCTGGGCAACAGAGTGAGACCCGTGTCAACCAAAAAACAAAAAAACAAAAAAATTGATAATTGTTTTCTTCAGACATTTTCCGGGTT-----													
H.sapiens	GGTC													
P.troglod.	GGTC													
G.gorilla	GGTC													
P.abelii	----													

Immediate promoter

	-480	-470	-460	-450	-440	-430	-420	-410	-400	-390	-380	-370	-360	-350
H.sapiens	GTCTGAGGTTTTTGTGTTGGTCTTTACCTTTTTTTTTTTTTTTTTTTTTTTAAGTAGGAGTCTCATTCTGTTGCCCAGGCTGGAGTGCAGTGGCATGATCTTGGCTCCTGCAACCTCCGCTCCTGGGTTCAAGTGATTCT													
P.troglod.	-----TTTTTTTTTTTTTTTTTTTTTTGAGACAGAGTCTCATTCTGTTGCCCAGGAGGAGCAGTGGCATGATCTTGGCTCACTGCAAGCTCCACCTCCCGGGTTTCATGCCATTCT													
G.gorilla	-----													TCT
P.abelii	-----													TCT
	-340	-330	-320	-310	-300	-290	-282	-273	-263	-253	-243	-233	-223	-213
H.sapiens	CCTGCCTCAGCTCCTCTGAGTAGCCGGGACTACAGGCGCATGCCACGATGCTGGCTAATTTT---TTGTATTTTATAGTAGAGATGGGGTTTCACCATGTTAGCTAGGAGGCTCTCGATCTCCTGACCTCGTGATC													CCCC
P.troglod.	CTTGCCTCAGCTCCTCTGAGTAGCTGGGACTACAGGCGCCTGCCACCATGCTCCGGCTAAAAAAATTGCATTTTATAGTAGAGATGGGGTTTCACCATGTTAGCCAGGATGCTCTCAATCTCCTGACCTCGTGATT													CCCC
G.gorilla	CCTGCCTCAGCTCCTCTGAGTAGCCGGGACTACAGGCGCATGCCACGATGCTGGCTAATTTT---TTGTATTTTATAGTAGAGATGGGGTTTCACCATGTTAGCCAGGAGGATCTCGATCTCCTGACCTCGTGATC													CCCC
P.abelii	CCTGCCTCAGCTCCTCTGAGTAGCCGGGACTACAGGCGCATGCCACGATGCTGGCTAATTTT---TTGTATTTTATAGTAGAGATGGGGTTTCACCATGTTAGCCAGGAGGATCTCGATCTCCTGACCTCGTGATC													CCCC
	-203	-193	-183	-173	-163	-153	-143	-133	-123	-113	-103	-93	-83	-73
H.sapiens	AdCTCGGCCCTCCCAAAGTGCTGGAATTACAGGTGTGAGCCACCACGCCCGGCCCTGATCTTTACATTTTAAATATTGCATTAGTGAACCGTGTACTGATTTTGTGATCATAGATAACCCAGTTAAATATTAAGTCTTAA													
P.troglod.	AdCTCAGCCTCCCAAAGTGCTGGGATTACAGGCAATGAGCCACCACACCCGGCCCT-----													
G.gorilla	GdCTCGGCCCTCCCAAAGTGCTGGAATTACAGGTGTGAGCCACCACGCCCGGCCCTGATCTTTACATTTTAAATATTGCATTAGTGAACCGTGTACTGATTTTGTGATCATAGATAACCCGGTTAAATATTAAGTCTTAA													
P.abelii	GdCTCGGCCCTCCCAAAGTGCTGGAATTACAGGTGTGAGCCACCACGCCCGGCCCTGATCTTTACATTTTAAATATTGCATTAGTGAACCGTGTACTGATTTTGTGATCATAGATAACCCGGTTAAATATTAAGTCTTAA													
	-63	-53	-43	-33	-23	-13	-3	7	17	27	37	47	57	67
H.sapiens	TTATCACTTAGTATTTTACAACCTCAGTTGCAGTTATAAAGTAAGGGTTCCACATACCTCCTAACAGTTCCTAGAAAAATGGAGCTGTCTTGGCATGTAGTCTTTATTTGCCCTGCTAAGTTTTTCATGCTGGGGGTCAGAC													
P.troglod.	-----													
G.gorilla	TTATCACTTAGTATTTTACAACCTCAGTTGTAGTTATAAAGTAAGGGTTCCACATACCTCCTAACAGTTCCTAGAAAAATGGAGCTGTCTTGGCATGTAGTCTTTATTTGCCCTGCTAAGTTTTTCATGCTGGGGGTCAGAC													
P.abelii	TTATCACTTAGTATTTTACAACCTCAGTTGTAGTTATAAAGTAAGGGTTCCACATACCTCCTAACAGTTCCTAGAAAAATGGAGCTGTCTTGGCATGTAGTCTTTATTTGCCCTGCTAAGTTTTTCATGCTGGGGGTCAGAC													
	77	87	97	107	117	127	137	147	157	167	177	187		
H.sapiens	TGGGAGTCTGATAGAAATTTTCATTTCCACCGCTGGTCCCTCTAACCAATGACTTGCTGCACAACCTGAGTGGTCTCCTGGGAGACCAGAGTTCTAACTTTGTAGCAGGGGACAAAGACA													
P.troglod.	-----													
G.gorilla	TGGGAGTCTGATAGAAATTTTCATTTCCACCGCTGGTCCCTCTAACCAATGACTTGCTGCACAACCTGAGTGGTCTCCTGGGAGACCAGAGTTCTAACTTTGTAGCAGGGGACAAAGACA													
P.abelii	TGGGAGTCTGATAGAAATTTTCATTTCCACCGCTGGTCCCTCTAACCAATGACTTGCTGCACAACCTGAGTGGTCTCCTGGGAGACCAGAGTTCTAACTTTGTAGCAGGGGACAAAGACA													

Intron2, *LCT*

	5290	5300	5310	5320	5330	5340	5350	5360	5370	5380	5390	5400	5410	5420
H.sapiens	CTGGGAAGTGAACAGCTTTGGGCCCCCTTGGGCAGCTGGGCAGTCTGGAAGCCATGGGAACCAACCGCGGTCTCCGTAGTTCTGCAGCATGGCCCTGGTGTGTAGAGATGTTGTGCTGCTCATTGGCGTCTGTGCGTTG													
P.troglod.	CTGGGAAGTGAACAGCTTTGGGCCCCCTTGGGCAGCTGGGCAGTCTGGAAGCCATGGGAACCAACCGCGGTCTCCGTAGTTCTGCAGCATGGCCCTGGTGTGTAGAGATGTTGTGCTGCTCATTGGCGTCTGTGCGTTG													
G.gorilla	CTAGGAAGTGAACAGCTTTGGGCCCCCTTGGGCAGCTGGGCAGTCTGGGAGCCATGGGAACCACTGCTGGTCTCCGTAGTTCTGCAGCATGGCCCTGGTGTGTAGAGATGTTGTGCTGCTCATTGGCGTCTGTGCGTTG													
P.abelii	CTGGGAAGTGAACGGCTTCAGGCCCTTGGGCAGCTGGGCAGTCTGGAAGCCATGGGAACCACTGCTGGTCTCCGTAGTTCTGCAGCATGGCTCTGGCACTGAAGAGATGCCGTGCTGCTCATTGGCGTCTGTGTGTTG													
	5430	5440	5450	5460	5470	5480	5489	5499	5509	5519	5529	5539	5549	5559
H.sapiens	ATGGGGGCCCCAGGAGTCAGGGTCTGGTCTTCAGATTTTCCATTTCAAACACCGATGGAACCAAGAC-TGATAAGGTTCTGGAGGGACAACCTCCAGGCTGATTGCACGGCCAGAATGGCACCTAAATTAGTTTACAGAGG													
P.troglod.	ATGGGGGCCCCAGGAGTCAGGGTCTGGTCTTCAGATTTTCCAGTTCAAACACCGATGGAACCAAGAC-TGATAAGGTTCTGGAGGGACAACCTCCAGGCTGATTGCACGGCCAGAATGGCACCTAAATTAGTTTTCAGAGG													
G.gorilla	ATGGGGGCCCCAGGAGTCAGGGTCTGGTCTTCAGATTTTCCAGTTCAAACACCGATGGAACCAAGAC-TGATAAGGTTCTGGAGGGACAACCTCCAGGCTGATTGCACGGCCAGAATGGCACCTAAATTAGTTTACAGAGG													
P.abelii	ATGGGGGCCCCAGGAGTCAGGGTCTGGTCTTCAGATTTTCCAGTTCAAACACCGATGGAACCAAGACCTGATAAGGTTCTGGAGGGACAACCTCCAGGCTGATTGCACGGCCAGAATGGCACCTAAATTAGTTTACAGAGG													
	5569	5579	5589	5599	5609	5619	5629	5639	5649	5659	5669	5679	5689	5699
H.sapiens	GCGATAACTGCTATCAGTTATGAAGCGCAGGAGACCAAAAGTGCTGATCAGAAAAATGCTTGTGGAGAAATAGCCTTTGTACTGGTCTCTCTGCCCCAGTCCATGCTGTGCAATGCTTCTGGGTCACTCTCCAAAAAGCAG													
P.troglod.	GCGATAACTGCTATCAGTTATGAAGCGCAGGAGACCAAAAGTGCTGATCAGAAAAATGCTTGTGGAGAAATAGCCTTTGTACTGGTCTCTCTGCCCCAGTCCATGCTGTGCAATGCTTCTGGGTCACTCTCCAAAAAGCAG													
G.gorilla	GCGATAACTGCTATCAGTTATGAAGCGCAGGAGACCAAAAGTGCTGATCAGAAAAATGCTTGTGGAGAAATAGCCTTTGTACTGGTCTCTCTGCCCCAGTCCATGCTGTGCAATGCTTCTGGGTCACTCTCCAAAAAGCAG													
P.abelii	GCGATAACTGCTATCAGTTATGAAGCGCGGAGACCAAAAGTGCTGATCAGAAAAATGCTTGTGGAGAAATAGCCTTTGTACTAGTCTCTCCGCCCACTCCATGCTGTGCAACGCTTCTGGGTAAATGTCCAAAAAGCAG													
	5709	5719	5729	5739	5749	5759	5769	5779	5789	5799	5809	5819	5829	5839
H.sapiens	TTCTGACCAGTTACCTGCTGGGAAGCCACACTTCCGAGTGGGACGAAATCCACAGGACCCCTCCAGTGCATGAACGTGTGGGGTCTCTCTCTCTCTCTCTGCGCTCACTCTCTATTTTGTCCATGTGAGTGTCTATGCT													
P.troglod.	TTCTGACCAGTTACCTGCTGGGAAGCCACACTTCTGAGTGGGATGAATCCACAGGACCCCTCCAGTGCATGAACGTGTGGGGTCTCTCTCTCTCTCTCTGCGCTCACTCTCTATTTTGTCCACGTGAGTGTCTATGCT													
G.gorilla	TTCTGACCAGTTACCTGCTGGGAAGCCACACTTCCGAGTGGGACGAAATCCACAGGACCCCTCCAGTGCATGAACGTGTGGGGTCTCTCTCTCTCTCTCTGCGCTCACTCTCTATTTTGTCCATGTGAGTGTCTATGCT													
P.abelii	TTCTGACCAGTTCCCTGCTGGGAAGCCACACTCCCGAGTGGGACGAAATCCCGCAGGACCCCTCCAGTGCATGAATATGTGGGGTCTCTCTCTCTCTCTCTGCGCTCACTCTCTATTTTGTCCCGTGTGAGTGTCTATGCT													
	5849	5859	5869	5879	5889	5891	5901	5911	5921	5931	5941	5951	5961	5971
H.sapiens	TGCTTCTACCTCCTGCTATCCCTACTGGAGCTCCTTCTGTCTTCTCTG-----CCTTCTCAAATCTCCTTCCAGCAAAATTTTCTCCATTGTTAATGTACACGTAACTGTGCTCTCCAGCTGAGGCCTCGCTCA													
P.troglod.	TGCTTCTACCTCCTGCTATCCCTACTGGAGCTCCTTCTGTCTTCTCTG-----CCTTCTCAAATCTCCTTCCAGCAAAATTTTCTCCATTGTTAATGTACACGTAACTGTGCTCTCCAGCTGAGGCCTCGCTCA													
G.gorilla	TGCTTCTACCTCCTGCTATCCCTACTGGAGCTCCTTCTGTCTTCTCTG-----CCTTCTCAAATCTCCTTCCAGCAAAATTTTCTCCATTGTTAATGTACACGTAACTGTGCTCTCCAGCTGAGGCCTCGCTCA													
P.abelii	TGCTTCTACCTCCTGCTATCCCTACTGGAGCTCCTTCTGTCTTCTCTGTTTGGCTTCTCTCAAATCTCCTTCCAGCAAAATTTTCTCCATTGTTAATGTACATGTAACGTGTGCTCTCCAGCGGAGGCCTCACTCA													
	5981	5991	6001	6011	6021	6031	6041	6051	6061	6071	6081	6091	6101	6111
H.sapiens	GTAACACTCCCTCACTCACCCTGATGCACTCTGTCCCCACCGGACCCCTTAGCCATTGCTTTAGACCAGTGGTTCCCAAGCTTGAGTGGGCATCAGAACCACCTAGAGAGCTAGGAGAGAGCACGGAGACCAAGACTTAA													
P.troglod.	GTAACACTCCCTCACTCACCCTGGTGCACCTCTGTCCCCACCGGACCCCTTAGCCATTGCTTTAGACCAGTGGTTCCCAAGCTTGAGTGGGCATCAGAACCACCTAGAGAGCTAGGAGAGAGCACGGAGACCAAGACTTAA													
G.gorilla	GTAACACTCCCTCACTCACCCTGATGCACTCTGTCCCCACCGGACCCCTTAGCCATTGCTCTAGACCAGTGGTTGCCAAGCTTGAGTGGGCATCAGAACCACCTAGAGAGCTAGGAGAGAGCACAGAGACCAAGACTTAA													
P.abelii	GTAACACTCCCTCACTCACCCTGATGCGCTCTCTCCCCACCGGACCCCTTAGCCATTGCTTTAGACCAGTGTTCCTCCCAAGCTTGAGTGGGCATCAGAACCACCTAGAGAGCTAGGAGAGAGCACAGAGACCAAGACTTAA													
													
H.sapiens	TGTGGTGG													
P.troglod.	TGTGGTGG													
G.gorilla	TGTGGTGG													
P.abelii	TGTGGTGG													

Appendix B1: Frequencies of lactase persistence associated alleles, - indicates that not genotyped at that position.

Continent/Region	Country	Population/Region	Longitude	Latitude	Number of chromosomes	-14010 G>C	-14009 T>G	-13915 T>G	-13910 C>T	-13907 C>G	Sum of all LP associated alleles	Reference
Africa	Algeria	Algerian	-1.32	34.88	21	-	-	-	0.33	-	0.33	Mulcare (2006) PhD thesis, UCL
Africa	Algeria	Berber Mzab	3.68	32.49	66	0.00	0.00	0.00	0.17	0.00	0.17	Myles et al. (2005) Hum Genet. 117, 34.
Africa	Algeria	Mozabite	3.68	32.49	60	-	-	-	0.22	-	0.22	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Africa	Angola	Kuvale	12.15	-15.20	108	0.06	0.00	0.00	0.00	0.00	0.06	Coelho et al. (2009) BMC Evol Biol. 9, 80.
Africa	Angola	Nyaneka-Nkhumbi	12.15	-15.20	306	0.03	0.00	0.00	0.00	0.00	0.03	Coelho et al. (2009) BMC Evol Biol. 9, 80.
Africa	Angola	Ovimbundu	12.15	-15.20	192	0.01	0.00	0.00	0.00	0.00	0.01	Coelho et al. (2009) BMC Evol Biol. 9, 80.
Africa	Cameroon	Fulani	14.15	11.05	102	0.00	0.00	0.00	0.39	0.00	0.39	Ingram et al. (2007) Hum Genet. 120, 779, Ingram (2008) PhD thesis UCL
Africa	Cameroon	Fulbe	14.15	11.05	102	-	-	-	0.21	-	0.21	Coelho et al. (2005) Hum Genet. 117, 329
Africa	Cameroon	Hausa	11.55	6.47	36	-	-	-	0.14	-	0.14	Mulcare et al. (2004) Am J Hum Genet. 74, 1102.
Africa	Cameroon	Mambila	12.50	6.00	134	0.00	0.00	0.00	0.00	0.00	0.00	Jones (2012) PhD thesis, UCL, unpublished data.
Africa	Cameroon	Nso	10.67	6.20	252	-	-	-	0.00	-	0.00	Mulcare et al. (2004) Am J Hum Genet. 74, 1102.
Africa	Cameroon	Pygmy	14.75	2.92	36	0.00	0.00	0.00	0.00	0.00	0.00	Jones (2012) PhD thesis, UCL, unpublished data.
Africa	Cameroon	Shuwa Arabs	14.50	13.00	124	0.00	0.00	0.08	0.02	0.00	0.10	Jones (2012) PhD thesis, UCL, unpublished data.
Africa	Cameroon	Yamba	11.55	6.47	42	-	-	-	0.00	-	0.00	Mulcare et al. (2004) Am J Hum Genet. 74, 1102.
Africa	Congo	Brazzaville	15.28	-4.27	104	0.00	0.00	0.00	0.00	0.00	0.00	Jones (2012) PhD thesis, UCL, unpublished data.
Africa	Ethiopia	Afar	41.36039	11.60212	152	0.01	0.01	0.18	0.01	0.24	0.45	Jones (2012) PhD thesis, UCL, unpublished data.
Africa	Ethiopia	Amhara	38.65951	9.869192	152	0.00	0.03	0.04	0.00	0.06	0.13	Jones (2012) PhD thesis, UCL, unpublished data.
Africa	Ethiopia	Anuak	34.41219	7.953241	138	0.00	0.00	0.00	0.00	0.00	0.00	Jones (2012) PhD thesis, UCL, unpublished data.
Africa	Ethiopia	Maale	36.64333	5.714812	132	0.00	0.05	0.04	0.00	0.02	0.11	Jones (2012) PhD thesis, UCL, unpublished data.
Africa	Ethiopia	Manjo	36.23	7.27	80	0.00	0.03	0.00	0.00	0.01	0.04	Jones (2012) PhD thesis, UCL, unpublished data.
Africa	Ethiopia	Nuer	34.58	8.25	74	0.00	0.00	0.00	0.00	0.00	0.00	Jones (2012) PhD thesis, UCL, unpublished data.
Africa	Ethiopia	Oromo	37.30817	7.83736	152	0.01	0.03	0.07	0.00	0.07	0.18	Jones (2012) PhD thesis, UCL, unpublished data.
Africa	Ethiopia	Shabo	35.41	7.56	48	0.00	0.00	0.00	0.00	0.00	0.00	Jones (2012) PhD thesis, UCL, unpublished data.

Africa	Ethiopia	Somali	41.87	9.58	186	0.01	0.02	0.05	0.02	0.06	0.16	Jones (2012) PhD thesis, UCL, unpublished data,
Africa	Ethiopia	Somali (phenotyped)	41.87	9.58	218	0.00	0.01	0.05	0.02	0.06	0.15	Ingram (2009) J Mol Evol. 69, 579.
Africa	Ethiopia	Suri	35.59	7.00	100	0.04	0.00	0.00	0.00	0.00	0.04	Jones (2012) PhD thesis, UCL, unpublished data.
Africa	Ethiopia	Amhara (phenotyped)	38.65951	9.869192	108	0.00	0.05	0.06	0.00	0.02	0.12	Jones et al. (2013) Am J Hum Genet. 93, 538.
Africa	Ethiopia	Oromo (phenotyped)	37.30817	7.83736	150	0.01	0.03	0.13	0.00	0.05	0.23	Jones et al. (2013) Am J Hum Genet. 93, 538.
Africa	Ethiopia	Tigray	39.47	13.50	88	0.00	0.03	0.11	0.00	0.19	0.34	Jones et al. (2013) Am J Hum Genet. 93, 538.
Africa	Ethiopia	Wolayta	37.76	6.84	52	0.02	0.04	0.12	0.00	0.08	0.25	Jones et al. (2013) Am J Hum Genet. 93, 538.
Africa	Ghana	Akan	-1.02	7.95	392	0.00	0.00	0.00	0.00	0.00	0.00	Tornaiainen et al. (2009) BMC Genet. 10, 31.
Africa	Ghana	Asante	-0.55	6.17	70	0.00	0.00	0.00	0.00	0.00	0.00	Jones (2012) PhD thesis, UCL, unpublished data.
Africa	Ghana	Builsa	-1.29	10.53	42	0.00	0.00	0.00	0.00	0.00	0.00	Jones (2012) PhD thesis, UCL, unpublished data.
Africa	Kenya	Borana	37.91	-0.02	16	0.13	-	0.19	0.00	0.13	0.44	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Kenya	Burji	37.91	-0.02	16	0.06	-	0.00	0.00	0.00	0.06	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Kenya	El Molo	37.91	-0.02	18	0.11	-	0.00	0.00	0.00	0.11	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Kenya	Gabra	37.91	-0.02	18	0.00	-	0.28	0.00	0.11	0.39	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Kenya	Kikuyu	37.91	-0.02	4	0.75	-	0.00	0.00	0.00	0.75	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Kenya	Konso	37.91	-0.02	12	0.08	-	0.08	0.00	0.00	0.17	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Kenya	Maasai	37.91	-0.02	64	0.58	-	0.00	0.00	0.03	0.61	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Kenya	Marakwet	37.91	-0.02	14	0.36	-	0.07	0.00	0.00	0.43	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Kenya	Nandi	37.91	-0.02	8	0.25	-	0.00	0.00	0.00	0.25	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Kenya	Ogiek	37.91	-0.02	22	0.36	-	0.00	0.00	0.00	0.36	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Kenya	Pokot	37.91	-0.02	28	0.29	-	0.04	0.00	0.00	0.32	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Kenya	Rendille	37.91	-0.02	16	0.13	-	0.13	0.00	0.06	0.31	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Kenya	Sabaot	37.91	-0.02	12	0.17	-	0.00	0.00	0.00	0.17	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Kenya	Samburu	37.91	-0.02	18	0.28	-	0.06	0.00	0.06	0.40	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Kenya	Sengwer	37.91	-0.02	32	0.06	-	0.00	0.00	0.00	0.06	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Kenya	Somali	37.91	-0.02	2	0.00	-	0.50	0.00	0.00	0.50	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Kenya	Tugen	37.91	-0.02	32	0.19	-	0.00	0.00	0.00	0.19	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Kenya	Turkana	37.91	-0.02	26	0.21	-	0.00	0.00	0.00	0.21	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Kenya	Wata	37.91	-0.02	2	0.00	-	0.00	0.00	0.00	0.00	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Kenya	Yaaku	37.91	-0.02	28	0.54	-	0.00	0.00	0.04	0.58	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Malawi	Bantu	33.78	-13.98	310	-	-	-	0.00	-	0.00	Mulcare et al. (2004) Am J Hum Genet. 74, 1102.
Africa	Malawi	Chewa	33.78	-13.98	100	0.00	0.00	0.00	0.00	0.00	0.00	Jones (2012) PhD thesis, UCL, unpublished data.
Africa	Morocco	Berber	-3.77	34.05	154	-	-	-	0.14	-	0.14	Mulcare et al. (2004) Am J Hum Genet. 74, 1102.
Africa	Morocco	Moroccan	-6.84	34.03	180	-	-	-	0.18	-	0.18	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Africa	Morocco	Moroccan	-6.84	34.03	24	0.00	0.00	0.08	0.21	0.00	0.29	Enattah et al. (2008) Am J Hum Genet. 82, 57.
Africa	Morocco	Saharawi	-6.84	34.03	114	-	-	-	0.26	-	0.26	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Africa	Morocco	Saharawi	-6.84	34.03	22	0.00	0.00	0.18	0.23	0.00	0.41	Enattah et al. (2008) Am J Hum Genet. 82, 57.
Africa	Morocco	Berber Amizmiz (High-Atlas)	-8.23	31.21	78	0.00	0.00	0.00	0.14	0.00	0.14	Myles et al. (2005) Hum Genet. 117, 34.
Africa	Morocco	Berber Moyen-Atlas (Mid-Atlas)	-4.84	33.12	66	0.00	0.00	0.00	0.16	0.00	0.16	Myles et al. (2005) Hum Genet. 117, 34.

Africa	Mozambique	Mozambique (Maputo, Ronga Bantu)	32.58	-25.97	94	-	-	-	0.01	-	0.01	Coelho et al. (2005) Hum Genet. 117, 329
Africa	Mozambique	Sena	35.05	-17.44	136	0.00	0.00	0.00	0.00	0.00	0.00	Jones (2012) PhD thesis, UCL, unpublished data.
Africa	N.E. Kenya	Bantu	35.49	3.32	24	-	-	-	0.00	-	0.00	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Africa	Namibia	San	18.19	-25.60	14	-	-	-	0.00	-	0.00	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Africa	Namibia	San	18.19	-25.60	34	0.06	0.00	0.00	0.00	0.00	0.06	Jones (2012) PhD thesis, UCL, unpublished data.
Africa	Nigeria	Yoruba	3.47	7.23	50	-	-	-	0.00	-	0.00	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Africa	São Tomé and Príncipe	Sao Tome	6.73	0.34	284	-	-	-	0.05	-	0.05	Coelho et al. (2005) Hum Genet. 117, 329
Africa	Senegal	Mandjak	-15.88	12.986	114	0.00	0.00	0.00	0.00	0.00	0.00	Jones (2012) PhD thesis, UCL, unpublished data.
Africa	Senegal	Wolof	-17.453	14.687	138	0.00	0.00	0.00	0.00	0.00	0.00	Jones (2012) PhD thesis, UCL, unpublished data.
Africa	Somalia	Somali	45.37	2.07	158	-	-	-	0.03	-	0.03	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Africa	South Africa	Bantu	28.08	-26.20	16	-	-	-	0.00	-	0.00	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Africa	South Africa	Mixed (for example with Europeans)	18.42	-33.92	124	0.07	0.00	0.00	0.22	0.00	0.28	Torniaainen et al. (2009) BMC Genet. 10, 31.
Africa	South Africa	South Africans	28.23	-25.71	40	0.08	0.00	0.00	0.00	0.00	0.08	Jones (2012) PhD thesis, UCL, unpublished data.
Africa	South Africa	Xhosa	20.66	-34.16	218	0.13	0.00	0.00	0.00	0.00	0.13	Torniaainen et al. (2009) BMC Genet. 10, 31.
Africa	Sudan	Ama	30.22	12.86	4	0.00	-	0.00	0.00	0.00	0.00	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Sudan	Beja (Banuamir)	30.22	12.86	12	0.00	-	0.17	0.00	0.25	0.42	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Sudan	Beja (Hadandawa)	30.22	12.86	22	0.00	-	0.09	0.00	0.18	0.27	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Sudan	Beni Amer	37.22	19.62	170	0.00	0.11	0.24	0.01	0.06	0.42	Jones (2012) PhD thesis, UCL, unpublished data.
Africa	Sudan	Dinka	30.22	12.86	18	0.00	-	0.00	0.00	0.00	0.00	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Sudan	Dinka	30.22	12.86	68	-	-	-	0.00	-	0.00	Mulcare et al. (2004) Am J Hum Genet. 74, 1102.
Africa	Sudan	Dunglawi	30.00	20.00	12	0.00	0.00	0.00	0.00	0.08	0.08	Ingram et al. (2007) Hum Genet. 120, 779, Ingram (2008) PhD thesis UCL
Africa	Sudan	Fulani	30.00	20.00	88	-	-	-	0.48	-	0.48	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Africa	Sudan	Gaali	32.53	15.59	20	0.00	0.00	0.00	0.00	0.05	0.05	Enattah et al. (2008) Am J Hum Genet. 82, 57.
Africa	Sudan	Jaali	33.43	16.69	172	0.00	0.06	0.13	0.01	0.01	0.21	Ingram et al. (2007) Hum Genet. 120, 779, Ingram (2008) PhD thesis UCL
Africa	Sudan	Koalib	30.22	12.86	2	0.00	-	0.00	0.00	0.00	0.00	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Sudan	Liguri/Logorik	30.22	12.86	2	0.00	-	0.00	0.00	0.00	0.00	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Sudan	Mahas	32.53	15.59	30	0.00	0.00	0.17	0.00	0.00	0.17	Enattah et al. (2008) Am J Hum Genet. 82, 57.
Africa	Sudan	Masalit	30.22	12.86	2	0.00	-	0.00	0.00	0.00	0.00	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Sudan	Nuer	30.22	12.86	10	0.00	-	0.00	0.00	0.00	0.00	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Sudan	Shaigi	30.00	20.00	18	0.00	0.17	0.06	0.00	0.00	0.22	Ingram et al. (2007) Hum Genet. 120, 779, Ingram (2008) PhD thesis UCL
Africa	Sudan	Shilook	30.22	12.86	16	0.00	-	0.00	0.00	0.00	0.00	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Tanzania	Akie	34.89	-6.37	28	0.25	-	0.00	0.00	0.00	0.25	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Tanzania	Burunge	34.89	-6.37	36	0.38	-	0.00	0.00	0.00	0.38	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Tanzania	Chagga	38.05	-5.38	92	0.14	0.00	0.00	0.00	0.00	0.14	Jones (2012) PhD thesis, UCL, unpublished data.
Africa	Tanzania	Datog	34.89	-6.37	8	0.63	-	0.00	0.00	0.00	0.63	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Tanzania	Dorobo	34.89	-6.37	20	0.40	-	0.00	0.00	0.00	0.40	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Tanzania	Fiome	34.89	-6.37	24	0.55	-	0.00	0.00	0.00	0.55	Tishkoff et al. (2007) Nat Genet. 39, 31

Africa	Tanzania	Hadza	34.89	-6.37	36	0.00	-	0.00	0.00	0.00	0.00	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Tanzania	Iraqw	34.89	-6.37	78	0.58	-	0.00	0.00	0.00	0.58	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Tanzania	Maasai	34.89	-6.37	38	0.45	-	0.00	0.00	0.00	0.45	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Tanzania	Mbugu	34.89	-6.37	60	0.31	-	0.00	0.00	0.00	0.31	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Tanzania	Mbugwe	34.89	-6.37	26	0.27	-	0.04	0.00	0.00	0.31	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Tanzania	Pare	34.89	-6.37	20	0.10	-	0.00	0.00	0.00	0.10	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Tanzania	Rangi	34.89	-6.37	70	0.27	-	0.00	0.00	0.00	0.27	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Tanzania	Samba'a	34.89	-6.37	6	0.00	-	0.00	0.00	0.00	0.00	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Tanzania	Sandawe	34.89	-6.37	62	0.13	-	0.00	0.00	0.00	0.13	Tishkoff et al. (2007) Nat Genet. 39, 31
Africa	Uganda	Bantu	32.98	0.43	44	-	-	-	0.00	-	0.00	Mulcare et al. (2004) Am J Hum Genet. 74, 1102.
Asia	Afghanistan	Pashtuns/Afghans	68.36	33.56	32	0.00	0.00	0.00	0.06	0.00	0.06	Liebert (2013) this thesis
Asia	Afghanistan	Tadjiks	68.71	36.13	28	0.00	0.00	0.00	0.25	0.00	0.25	Liebert (2013) this thesis
Asia	Afghanistan	Tadjiks	68.71	36.13	80	-	-	-	0.08	-	0.08	Mulcare (2006) PhD thesis, UCL
Asia	Afghanistan	Uzbeks	67.64	35.50	54	0.00	0.00	0.00	0.24	0.00	0.24	Liebert (2013) this thesis
Asia	Afghanistan	Uzbeks	67.64	35.50	30	-	-	-	0.03	-	0.03	Mulcare (2006) PhD thesis, UCL
Asia	Armenia	Armenians	45.04	40.07	88	-	-	-	0.01	-	0.01	Mulcare (2006) PhD thesis, UCL
Asia	Armenia	Armenians	45.04	40.07	102	0.00	0.00	0.00	0.18	0.00	0.18	Liebert (2013) this thesis
Asia	Azerbaijan	Azeri	47.58	40.14	80	0.00	0.00	0.00	0.01	0.00	0.01	Liebert (2013) this thesis, Mulcare (2006) PhD thesis, UCL
Asia	Cambodia	Cambodian	104.92	11.55	22	-	-	-	0.00	-	0.00	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Asia	China	Dai	102.83	24.88	20	-	-	-	0.00	-	0.00	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Asia	China	Daur	87.62	43.83	20	-	-	-	0.05	-	0.05	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Asia	China	Han	104.21	34.86	90	-	-	-	0.00	-	0.00	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Asia	China	Han	104.21	34.86	200	-	-	-	0.00	-	0.00	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Asia	China	Hezhen	132.50	47.70	20	-	-	-	0.00	-	0.00	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Asia	China	Hezhen	132.50	47.70	154	-	-	-	0.00	-	0.00	Sun et al. (2007) Asia Pac J Clin Nutr. 16, 598.
Asia	China	Hezhen	132.50	47.70	196	0.00	-	0.00	-	0.00	0.00	Xu et al. (2010) Scand J Gastroentero. 45, 168.
Asia	China	Kazak	81.30	43.90	188	-	-	-	0.05	-	0.05	Sun et al. (2007) Asia Pac J Clin Nutr. 16, 598.
Asia	China	Kazak	81.30	43.90	194	0.00	-	0.00	-	0.00	0.00	Xu et al. (2010) Scand J Gastroentero. 45, 168.
Asia	China	Lahu	102.83	24.88	20	-	-	-	0.00	-	0.00	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Asia	China	Man	123.20	40.20	130	-	-	-	0.00	-	0.00	Sun et al. (2007) Asia Pac J Clin Nutr. 16, 598.
Asia	China	Man (Manchu)	123.20	40.20	216	0.00	-	0.00	-	0.00	0.00	Xu et al. (2010) Scand J Gastroentero. 45, 168.
Asia	China	Miaozi	106.63	26.65	20	-	-	-	0.00	-	0.00	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Asia	China	Mongols	119.70	29.20	164	-	-	-	0.02	-	0.02	Sun et al. (2007) Asia Pac J Clin Nutr. 16, 598.
Asia	China	Mongols	119.70	29.20	212	0.00	-	0.00	-	0.00	0.00	Xu et al. (2010) Scand J Gastroentero. 45, 168.
Asia	China	Mongols	119.70	29.20	20	-	-	-	0.10	-	0.10	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Asia	China	Naxi	100.23	26.86	20	-	-	-	0.00	-	0.00	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Asia	China	Northern Han	126.53	45.80	138	0.00	-	0.00	-	0.00	0.00	Xu et al. (2010) Scand J Gastroentero. 45, 168.
Asia	China	Oroqen	131.00	53.50	20	-	-	-	0.00	-	0.00	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Asia	China	Oroqen	131.00	53.50	90	-	-	-	0.01	-	0.01	Sun et al. (2007) Asia Pac J Clin Nutr. 16, 598.
Asia	China	Oroqen	131.00	53.50	136	0.00	-	0.00	-	0.00	0.00	Xu et al. (2010) Scand J Gastroentero. 45, 168.
Asia	China	She	119.65	27.09	20	-	-	-	0.00	-	0.00	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Asia	China	Tibetans (Nagqu)	92.07	31.48	418	0.00	0.00	0.00	0.00	0.00	0.00	Peng et al. (2012) J Hum Genet. 57, 394.

Asia	China	Tibetans (Shigatse)	88.88	29.27	572	0.00	0.00	0.00	0.00	0.00	0.00	Peng et al. (2012) J Hum Genet. 57, 394.
Asia	China	Tu	101.78	36.62	20	-	-	-	0.00	-	0.00	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Asia	China	Tujia	112.94	28.23	20	-	-	-	0.00	-	0.00	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Asia	China	Uygur	87.62	43.83	20	-	-	-	0.05	-	0.05	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Asia	China	Xibo	123.43	41.81	18	-	-	-	0.00	-	0.00	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Asia	China	Yizu	103.77	29.55	20	-	-	-	0.00	-	0.00	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Asia	Georgia	Georgians	43.35	42.32	108	0.00	0.00	0.00	0.08	0.00	0.08	Liebert (2013) this thesis
Asia	India	Andaman and Nicobar Islands	92.77	11.67	68	0.00	0.00	0.00	0.02	0.00	0.02	Galego Romero et al. (2012) Mol Biol Evol. 29, 249.
Asia	India	Central	79.45	23.84	358	0.00	0.00	0.00	0.06	0.00	0.06	Galego Romero et al. (2012) Mol Biol Evol. 29, 249.
Asia	India	Dawoodi Bohra (Gujarat)	71.19	22.26	100	0.00	0.00	0.00	0.11	0.00	0.11	Easwaarkanth et al. (2009) Eur J Hum Genet. 18, 354
Asia	India	Dawoodi Bohra (Tamil Nadu)	78.66	11.13	124	0.00	0.00	0.00	0.14	0.00	0.14	Easwaarkanth et al. (2009) Eur J Hum Genet. 18, 354
Asia	India	East	85.02	20.80	620	0.00	0.00	0.00	0.02	0.00	0.02	Galego Romero et al. (2012) Mol Biol Evol. 29, 249.
Asia	India	Indian Shia	80.10	27.57	142	0.00	0.00	0.00	0.10	0.00	0.10	Easwaarkanth et al. (2009) Eur J Hum Genet. 18, 354
Asia	India	Indian Sunni	80.10	27.57	164	0.00	0.00	0.00	0.10	0.00	0.10	Easwaarkanth et al. (2009) Eur J Hum Genet. 18, 354
Asia	India	Iranian Shia	80.10	17.05	98	0.00	0.00	0.00	0.04	0.00	0.04	Easwaarkanth et al. (2009) Eur J Hum Genet. 18, 354
Asia	India	Mappla	78.66	11.13	124	0.00	0.00	0.00	0.02	0.00	0.02	Galego Romero et al. (2012) Mol Biol Evol. 29, 249.
Asia	India	North	76.78	30.75	580	0.00	0.00	0.00	0.16	0.00	0.16	Galego Romero et al. (2012) Mol Biol Evol. 29, 249.
Asia	India	North East	95.00	27.48	278	0.00	0.00	0.00	0.01	0.00	0.01	Liebert (2013) this thesis, Mulcare (2006) PhD thesis, UCL
Asia	India	North	77.20	28.60	120	0.00	0.00	0.00	0.20	0.00	0.20	Babu et al. (2010) Am J Clin Nutr. 91, 140
Asia	India	North (Lucknow)	80.95	26.85	154	0.00	0.00	0.00	0.19	0.00	0.19	Galego Romero et al. (2012) Mol Biol Evol. 29, 249.
Asia	India	South	77.57	12.97	1728	0.00	0.00	0.01	0.09	0.00	0.09	Liebert (2013) this thesis
Asia	India	South	80.28	13.08	102	0.00	0.00	0.00	0.12	0.00	0.12	Babu et al. (2010) Am J Clin Nutr. 91, 140
Asia	India	South-Indian (Bangalore)	77.59	12.97	152	0.00	0.00	0.00	0.07	0.00	0.07	Galego Romero et al. (2012) Mol Biol Evol. 29, 249.
Asia	India	West	73.68	24.58	936	0.00	0.00	0.00	0.21	0.00	0.21	Liebert (2013) this thesis
Asia	Mongolia	Khalka	99.05	47.38	114	0.00	0.00	0.00	0.03	0.00	0.03	Liebert (2013) this thesis
Asia	Mongolia	Mongols	106.92	47.92	52	0.00	0.00	0.00	0.04	0.00	0.04	Liebert (2013) this thesis
Asia	Nepal	Nepalese	84.12	28.39	38	0.00	0.00	0.00	0.34	0.00	0.34	Liebert (2013) this thesis
Asia	Nepal	Tharu	80.90	28.83	80	0.00	0.00	0.00	0.18	0.00	0.18	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Asia	Pakistan	Balochi	67.02	30.21	50	-	-	-	0.36	-	0.36	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Asia	Pakistan	Balti	74.59	35.95	46	-	-	-	0.00	-	0.00	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Asia	Pakistan	Baluch	65.09	28.49	38	-	-	-	0.34	-	0.34	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Asia	Pakistan	Brahui	67.02	30.21	50	-	-	-	0.34	-	0.34	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Asia	Pakistan	Brahui	67.02	30.21	60	-	-	-	0.27	-	0.27	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Asia	Pakistan	Burusho	74.59	35.95	50	-	-	-	0.10	-	0.10	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Asia	Pakistan	Burusho	74.59	35.95	60	-	-	-	0.02	-	0.02	

Asia	Pakistan	Hazara	67.02	30.21	28	-	-	-	0.04	-	0.04	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Asia	Pakistan	Hazara	67.02	30.21	50	-	-	-	0.08	-	0.08	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Asia	Pakistan	Kalash	71.78	35.84	60	-	-	-	0.00	-	0.00	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Asia	Pakistan	Kalash	71.78	35.84	50	-	-	-	0.00	-	0.00	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Asia	Pakistan	Kashmiri	73.94	33.89	40	-	-	-	0.12	-	0.12	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Asia	Pakistan	Makrani	67.02	30.21	50	-	-	-	0.34	-	0.34	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Asia	Pakistan	Makrani Baluch	68.03	26.27	58	-	-	-	0.17	-	0.17	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Asia	Pakistan	Mohannes	68.00	26.00	58	-	-	-	0.28	-	0.28	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Asia	Pakistan	Parsi	68.00	26.00	58	-	-	-	0.14	-	0.14	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Asia	Pakistan	Pathan	69.86	32.67	56	-	-	-	0.30	-	0.30	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Asia	Pakistan	Pathan	69.86	32.67	50	-	-	-	0.30	-	0.30	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Asia	Pakistan	Sindhi	67.03	24.89	50	-	-	-	0.32	-	0.32	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Asia	Pakistan	Sindhi	67.03	24.89	56	-	-	-	0.41	-	0.41	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Asia	Russia	Daghestans mixed	47.12	42.83	46	-	-	-	0.13	-	0.13	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Asia	Russia	Druss	47.12	42.83	34	-	-	-	0.12	-	0.12	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Asia	Russia	Nenets	49.02	67.66	178	-	-	-	0.07	-	0.73	Khabarova et al. (2012) Int J Circumpol Heal. 71, 1.
Asia	Russia	Nog	47.12	42.83	40	-	-	-	0.07	-	0.07	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Asia	Russia	North-Yakuts	146.19	70.77	22	0.00	0.00	0.00	0.14	0.00	0.14	Liebert (2013) this thesis
Asia	Russia	Ob-Ugric	68.00	65.00	40	-	-	-	0.03	-	0.03	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Asia	Russia	Udmurts	53.18	56.83	60	-	-	-	0.33	-	0.33	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Asia	Russia	Yakuts	129.73	62.03	50	-	-	-	0.06	-	0.06	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Asia	Russia	Yakuts	129.73	62.03	110	0.00	0.00	0.00	0.05	0.00	0.05	Liebert (2013) this thesis
Asia	Singapore	Han-Chinese	104.21	35.86	98	0.00	0.00	0.00	0.04	0.00	0.04	Liebert (2013) this thesis
Asia	Singapore	Japanese	138.24	36.21	84	0.00	0.00	0.00	0.00	0.00	0.00	Liebert (2013) this thesis
Asia	South Korea	South Korean	127.00	35.57	46	-	-	-	0.00	-	0.00	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Syria, Iraq, Lebanon,												
Asia	West Bank	Arabs	40.00	33.00	40	0.00	0.00	0.11	0.13	0.00	0.24	Enattah et al. (2008) Am J Hum Genet. 82, 57.
Asia	Uzbekistan	Kazakh	63.46	40.13	166	-	-	-	0.16	-	0.16	Heyer et al. (2011) Hum Biol. 83, 379.
Asia	Uzbekistan	Tajiko-Uzbek	64.42	39.77	200	-	-	-	0.10	-	0.10	Heyer et al. (2011) Hum Biol. 83, 379.
Asia	Uzbekistan	Uzbeks	64.59	41.38	76	0.00	0.00	0.00	0.04	0.00	0.04	Liebert (2013) this thesis
Australasia	Papua New Guinea	Papuan	140.71	-5.09	34	-	-	-	0.00	-	0.00	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Australasia	Solomon Islands	Melanesian (NAN)	155.19	-6.05	44	-	-	-	0.00	-	0.00	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Europe	Belarus	Belarusians	27.95	53.71	100	0.00	0.00	0.00	0.23	0.00	0.23	Liebert (2013) this thesis
Europe	England (Central)	British	-2.76	52.71	1368	-	-	-	0.76	-	0.76	Davey Smith et al. (2009) Eur J Hum Genet. 17, 357.
Europe	England (Northern)	British	-1.54	53.99	2336	-	-	-	0.75	-	0.75	Davey Smith et al. (2009) Eur J Hum Genet. 17, 357.
England												
Europe	(Southeastern)	British	-0.57	51.22	1894	-	-	-	0.70	-	0.70	Davey Smith et al. (2009) Eur J Hum Genet. 17, 357.
Europe	Estonia	Estonians	26.40	59.05	628	0.00	0.00	0.00	0.51	0.00	0.51	Lember (2006) World J Gastroentero. 12, 7329.
Europe	Finland	Finns	28.00	65.00	1876	0.00	0.00	0.00	0.58	0.00	0.58	Enattah et al. (2008) Am J Hum Genet. 82, 57.
Europe	Finland	Saami	26.19	68.26	60	-	-	-	0.17	-	0.17	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Europe	Finland and Sweden	Scandinavians	18.05	59.33	360	-	-	-	0.82	-	0.82	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Europe	Finns	Finns, eastern	29.00	65.00	154	-	-	-	0.55	-	0.55	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Europe	Finns	Finns, western	24.00	64.00	308	-	-	-	0.62	-	0.62	Enattah et al. (2007) Am J Hum Genet. 81, 615.

Europe	France	Basques	-1.45	43.40	48	-	-	-	0.67	-	0.67	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Europe	France	Basques	-1.45	43.40	170	-	-	-	0.66	-	0.66	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Europe	France	French	2.22	46.23	58	-	-	-	0.43	-	0.43	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Europe	France	French	2.22	46.23	34	-	-	-	0.34	-	0.34	Enattah et al. (2007) Am J Hum Genet. 81, 615.
												Liebert (2013) this thesis, Mulcare (2006) PhD
Europe	Germany	Germans	10.45	51.17	60	0.00	0.00	0.00	0.55	0.00	0.55	thesis, UCL
Europe	Germany	Sorbs	14.31	51.26	64	0.00	0.00	0.00	0.50	0.00	0.50	Liebert (2013) this thesis
Europe	Greece	Greeks	21.83	39.07	200	-	-	-	0.09	-	0.09	Anagnostou et al. (2009) Am J Hum Biol. 21, 217.
												Liebert (2013) this thesis, Mulcare (2006) PhD
Europe	Greece	Greeks	21.83	39.07	120	0.00	0.00	0.00	0.15	0.00	0.15	thesis, UCL
Europe	Hungary	Hungarian	20.17	46.25	220	-	-	-	0.62	-	0.62	Nagy et al. (2009) Eur J Clin Nutr. 63, 909.
Europe	Hungary	Hungarian	20.17	46.25	362	-	-	-	0.36	-	0.36	Nagy et al. (2011) Am J Phys Anthr. 145, 262.
												Liebert (2013) this thesis, Mulcare (2006) PhD
Europe	Ireland	Irish	-8.00	53.33	68	0.00	0.00	0.00	0.96	0.00	0.96	thesis, UCL
Europe	Italy	Central	12.48	41.90	196	-	-	-	0.11	-	0.11	Anagnostou et al. (2009) Am J Hum Biol. 21, 217.
Europe	Italy	Central-North	11.25	43.77	412	-	-	-	0.13	-	0.13	Anagnostou et al. (2009) Am J Hum Biol. 21, 217.
Europe	Italy	Italians	13.91	42.22	134	-	-	-	0.13	-	0.13	Coelho et al. (2005) Hum Genet. 117, 329
Europe	Italy	North Italian	9.68	45.70	28	-	-	-	0.36	-	0.36	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Europe	Italy	North-Eastern	12.33	45.44	438	-	-	-	0.24	-	0.24	Anagnostou et al. (2009) Am J Hum Biol. 21, 217.
												Ingram et al. (2007) Hum Genet. 120, 779, Ingram
Europe	Italy	S. European	12.48	41.90	66	0.00	0.00	0.00	0.09	0.00	0.09	(2008) PhD thesis UCL
Europe	Italy	Sardinian	9.12	39.22	306	-	-	-	0.07	-	0.07	Anagnostou et al. (2009) Am J Hum Biol. 21, 217.
Europe	Italy	Sardinian	9.12	39.22	56	-	-	-	0.07	-	0.07	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Europe	Italy	South Italians	16.25	39.30	200	-	-	-	0.05	-	0.05	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Europe	Italy	Southern	16.25	39.30	378	-	-	-	0.08	-	0.08	Anagnostou et al. (2009) Am J Hum Biol. 21, 217.
Europe	Italy	Tuscan	10.98	43.57	16	-	-	-	0.06	-	0.06	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Europe	Italy	Tyrolean Bozen	11.35	46.50	80	0.00	0.00	0.00	0.15	0.00	0.15	Liebert (2013) this thesis
Europe	Italy	Tyrolean Gadertal	11.92	46.72	76	0.00	0.00	0.00	0.42	0.00	0.42	Liebert (2013) this thesis
Europe	Italy	Tyrolean Vinschgau	10.77	46.63	102	0.00	0.00	0.00	0.36	0.00	0.36	Liebert (2013) this thesis
Europe	Macedonia	Macedonians	21.75	41.61	100	0.00	0.00	0.00	0.24	0.00	0.24	Liebert (2013) this thesis
												Ingram et al. (2007) Hum Genet. 120, 779, Ingram
Europe	Mixed	N. European	15.00	54.00	110	0.00	0.00	0.00	0.62	0.00	0.62	(2008) PhD thesis UCL
Europe	Netherlands	Frisians	5.80	53.20	58	0.00	0.00	0.00	0.81	0.00	0.81	Liebert (2013) this thesis
Europe	Norway	Norwegians	8.47	60.47	88	0.00	0.00	0.00	0.84	0.00	0.84	Liebert (2013) this thesis
Europe	Poland	Ashkenazi-Jews	21.00	52.25	96	-	-	-	0.08	-	0.08	Mulcare (2006) PhD thesis, UCL
Europe	Poland	Polish	19.15	51.92	400	-	-	-	0.46	-	0.46	Madry et al. (2010) Acta Biochim Pol. 57, 585
Europe	Poland	Polish	19.15	51.92	446	-	-	0.00	0.30	0.00	0.30	Płoszaj et al. (2011) Cent Eur J Biol. 6, 176.
Europe	Portugal	Portuguese	-8.22	39.40	96	0.00	0.00	0.00	0.44	0.00	0.44	Liebert (2013) this thesis
Europe	Portugal	Portuguese-Centre	-7.50	40.28	140	-	-	-	0.39	-	0.39	Manco et al. (2012) Ann Hum Biol. 40, 205.
Europe	Portugal	Portuguese-North	-8.42	41.55	128	-	-	-	0.38	-	0.38	Manco et al. (2012) Ann Hum Biol. 40, 205.
Europe	Portugal	Portuguese-North	-8.42	41.55	180	-	-	-	0.37	-	0.37	Coelho et al. (2005) Hum Genet. 117, 329
Europe	Portugal	Portuguese-South	-7.83	38.52	130	-	-	-	0.27	-	0.27	Manco et al. (2012) Ann Hum Biol. 40, 205.
Europe	Romania	Romanians	24.97	45.94	118	0.00	0.00	0.00	0.17	0.00	0.17	Liebert (2013) this thesis

Europe	Romania (Eastern Transylvania)	Seklers	25.79	45.86	130	-	-	-	0.40	-	0.40	Nagy et al. (2011) Am J Phys Anthr. 145, 262.
Europe	Russia	Erzya	43.58	54.21	60	-	-	-	0.27	-	0.27	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Europe	Russia	Erzya	43.58	54.21	42	0.00	0.00	0.00	0.14	0.00	0.14	Liebert (2013) this thesis
Europe	Russia	Moksha	44.07	54.24	32	0.00	0.00	0.00	0.41	0.00	0.41	Liebert (2013) this thesis
Europe	Russia	Moksha	44.07	54.24	60	-	-	-	0.28	-	0.28	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Europe	Russia	Russian	37.62	55.75	50	-	-	-	0.24	-	0.24	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Europe	Russia	Russians (Northwest)	40.53	64.53	298	0.00	0.00	0.00	0.39	0.00	0.39	Khabarova et al. (2009) World J Gastroentero. 15, 1849.
Europe	Russia	Russians Perm	56.25	58.01	46	0.00	0.00	0.00	0.24	0.00	0.24	Liebert (2013) this thesis
Europe	Russia (Komi republic)	Komi	54.83	63.86	20	-	-	-	0.15	-	0.15	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Europe	Russian (Caucasus)	Adygei	40.08	44.60	34	-	-	-	0.12	-	0.12	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Europe	Scotland	British	-3.78	56.00	1032	-	-	-	0.82	-	0.82	Davey Smith et al. (2009) Eur J Hum Genet. 17, 357.
Europe	Slovakia	Roma	19.70	48.67	64	0.00	0.00	0.00	0.06	0.00	0.06	Liebert (2013) this thesis
Europe	Slovakia	Roma	19.70	48.67	108	-	-	-	0.11	-	0.11	Mulcare (2006) PhD thesis, UCL
Europe	Spain	Catalans	1.52	41.59	58	0.00	0.00	0.00	0.26	0.00	0.26	Liebert (2013) this thesis
Europe	Spain	Spanish	-3.70	40.51	1718	-	-	-	0.39	-	0.39	Agueda et al. (2010) Cadif Tissue Int. 87, 14.
Europe	Spain	Spanish	-3.70	40.51	62	0.00	0.00	0.00	0.40	0.00	0.40	Liebert (2013) this thesis
Europe	Sweden	Sami	20.87	67.87	60	0.00	0.00	0.00	0.29	0.00	0.29	Liebert (2013) this thesis
Europe	Sweden	Swedes	15.01	60.03	784	-	-	-	0.74	-	0.74	Almon et al. (2007) Scand J Gastroenterol. 42, 165.
Europe	Sweden	Swedes	15.01	60.03	74	0.00	0.00	0.00	0.78	0.00	0.78	Liebert (2013) this thesis
Europe	UK	Ashkenazi-Jews	-0.30	51.51	38	0.00	0.00	0.00	0.11	0.00	0.11	Liebert (2013) this thesis
Europe	UK	English	-0.12	51.51	102	0.00	0.00	0.00	0.75	0.00	0.75	Liebert (2013) this thesis, Mulcare (2006) PhD thesis, UCL
Europe	UK	Orcadian (Orkney Islands)	-3.15	59.04	32	-	-	-	0.69	-	0.69	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Europe	Ukraine	Ukrainians	31.17	48.38	92	-	-	-	0.22	-	0.22	Mulcare (2006) PhD thesis, UCL
Europe	Ukraine	Ukrainians	31.17	48.38	74	0.00	0.00	0.00	0.26	0.00	0.26	Liebert (2013) this thesis
Middle East	Cyprus	Greek-Cypriots	33.02	34.71	120	0.00	0.00	0.00	0.01	0.00	0.01	Liebert (2013) this thesis
Middle East	Iran	Iranians	53.69	32.43	42	0.00	0.00	0.00	0.10	0.00	0.10	Enattah et al. (2008) Am J Hum Genet. 82, 57.
Middle East	Iran	Iranians	53.69	32.43	154	0.00	0.00	0.01	0.03	0.00	0.04	Liebert (2013) this thesis, Mulcare (2006) PhD thesis, UCL
Middle East	Iran	Iranians	53.69	32.43	42	-	-	-	0.10	-	0.10	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Middle East	Iran	Qashqai	52.53	29.62	20	-	-	-	0.05	-	0.05	Enattah et al. (2007) Am J Hum Genet. 81, 615.
Middle East	Israel	Ashkenazi-Jews	34.77	32.07	192	-	-	-	0.09	-	0.09	Raz et al. (2013) Gene. 519, 67.
Middle East	Israel	Bedouin	34.88	30.71	98	-	-	-	0.03	-	0.03	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Middle East	Israel	Bedouin Israeli	34.77	32.07	38	0.00	0.00	0.13	0.03	0.00	0.16	Ingram et al. (2007) Hum Genet. 120, 779, Ingram (2008) PhD thesis UCL
Middle East	Israel	Bedouin Arabs	34.77	32.07	302	-	-	0.28	0.02	-	0.02	Raz et al. (2013) Gene. 519, 67.
Middle East	Israel	Druze	34.77	32.07	96	-	-	-	0.02	-	0.02	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Middle East	Israel	Druze	34.77	32.07	28	0.00	0.00	0.11	0.04	0.00	0.14	Ingram et al. (2007) Hum Genet. 120, 779, Ingram (2008) PhD thesis UCL
Middle East	Israel	Iraqi Jews	34.77	32.07	192	-	-	-	0.04	-	0.04	Raz et al. (2013) Gene. 519, 67.

Middle East	Israel	Moroccan	34.77	32.07	192	-	-	-	0.09	-	0.09	Raz et al. (2013) Gene. 519, 67.
Middle East	Israel	Palestinian Arabs	35.20	31.90	102	-	-	-	0.04	-	0.04	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Middle East	Israel	Urban Arabs	34.77	32.07	84.00	0.00	0.01	0.01	0.02	0.00	0.05	Liebert (2013) this thesis
Middle East	Israel	Urban Arabs	34.77	32.07	162	0.00	0.01	0.06	0.00	0.00	0.06	Ingram et al. (2007) Hum Genet. 120, 779, Ingram (2008) PhD thesis UCL
Middle East	Israel/PAA	Palestinian Arabs	35.20	31.90	36	0.00	0.00	0.00	0.00	0.00	0.00	Ingram et al. (2007) Hum Genet. 120, 779, Ingram (2008) PhD thesis UCL
Middle East	Japan	Japanese	138.24	37.21	62	-	-	-	0.00	-	0.00	Bersaglieri et al. (2004) Am J Hum Genet. 74, 1111.
Middle East	Jordan	Jordanian	35.93	31.95	112	0.00	0.00	0.05	0.05	0.00	0.11	Enattah et al. (2008) Am J Hum Genet. 82, 57.
Middle East	Jordan	Jordanian	36.24	30.59	46	0.00	0.00	0.35	0.00	0.00	0.35	Ingram et al. (2007) Hum Genet. 120, 779, Ingram (2008) PhD thesis UCL
Middle East	Kurwait	Ajman	48.00	28.83	74	0.00	0.00	0.06	0.00	0.00	0.06	Hill et al. (2013) Am J Phys Anthr. (Epub ahead of print)
Middle East	Kuwait	Kuwaiti	47.48	29.31	66	0.00	0.02	0.25	0.03	0.00	0.30	Liebert (2013) this thesis
Middle East	Kuwait	Kuwaiti	47.48	29.31	28	-	-	-	0.00	-	0.00	Mulcare (2006) PhD thesis, UCL
Middle East	Kuwait	Mutran	47.94	29.24	58	0.00	0.00	0.55	0.00	0.03	0.58	Hill et al. (2013) Am J Phys Anthr. (Epub ahead of print)
Middle East	Oman	Arabs of Northern Oman	57.01	23.88	684	0.00	0.00	0.14	0.01	0.00	0.15	Al-Abri et al. (2012) Hum Biol. 84, 271.
Middle East	Oman	Dhofaris Arabs of										
Middle East	Oman	Southern Oman	54.43	18.40	420	0.00	0.00	0.72	0.00	0.00	0.72	Al-Abri et al. (2012) Hum Biol. 84, 271.
Middle East	Oman	Omani	58.50	23.53	104	0.00	0.00	0.77	0.03	0.00	0.80	Al-Abri et al. (2013) Oman Med J. 28, 341.
Middle East	Saudi Arabia	Bedouin	45.00	23.00	94	0.00	0.00	0.48	0.00	0.00	0.48	Ingram et al. (2007) Hum Genet. 120, 779, Ingram (2008) PhD thesis UCL
Middle East	Saudi Arabia	Central	46.72	24.71	180	0.00	0.00	0.61	0.00	0.00	0.61	Imtiaz et al. (2007) J Med Genet. 44, e89.
Middle East	Saudi Arabia	Eastern	50.68	22.30	164	0.00	0.00	0.62	0.00	0.00	0.62	Imtiaz et al. (2007) J Med Genet. 44, e89.
Middle East	Saudi Arabia	Northern	39.32	29.89	164	0.00	0.00	0.52	0.01	0.00	0.53	Imtiaz et al. (2007) J Med Genet. 44, e89.
Middle East	Saudi Arabia	Southern	44.13	17.49	184	0.00	0.00	0.58	0.00	0.00	0.58	Imtiaz et al. (2007) J Med Genet. 44, e89.
Middle East	Saudi Arabia	Western	39.82	21.42	172	0.00	0.00	0.65	0.01	0.00	0.65	Imtiaz et al. (2007) J Med Genet. 44, e89.
Middle East	Saudi Arabia	Arabs	45.00	23.00	248	0.00	0.00	0.57	0.00	0.01	0.58	Enattah et al. (2008) Am J Hum Genet. 82, 57.
Middle East	Syria	Assyrians	36.30	33.50	80	-	-	-	0.04	-	0.04	Mulcare (2006) PhD thesis, UCL
Middle East	Syria	Syrians	39.00	34.80	140	0.00	0.00	0.03	0.02	0.00	0.05	Liebert (2013) this thesis
Middle East	Turkey	Anatolian-Turks	31.33	38.99	98	-	-	-	0.03	-	0.03	Mulcare (2006) PhD thesis, UCL
Middle East	Turkey	Anatolian-Turks	31.33	38.99	116	0.00	0.00	0.00	0.08	0.00	0.08	Liebert (2013) this thesis
Middle East	Yemen	Yemeni_Hadramaut	49.37	16.93	166	0.01	0.00	0.24	0.02	0.01	0.28	Liebert (2013) this thesis
Middle East	Yemen	Yemeni_Sena	44.21	15.35	68	0.00	0.00	0.29	0.15	0.00	0.44	Liebert (2013) this thesis
Middle East	Yemen	Yemeni (Sana'a University)	44.21	15.35	478	0.00	0.00	0.55	0.00	0.00	0.55	Al-Abri et al. (2012) Hum Biol. 84, 271.

Appendix B2: Lactase persistence phenotype data. Test methods: BH: breath hydrogen, BG: blood glucose, UG: urine galactose.

Continent/ Region	Country	Population/ Region	Longitude	Latitude	N	Frequency of digesters	Test method	Reference
Africa	Botswana	Shua	28.63	-23.04	22.00	0.09	BG	Nurse & Jenkins (1974) Br Med J. 2, 728.
Africa	Egypt	Cairo and Giza	31.25	30.05	67.00	0.33	BH	Hussein et al. (1982) Hum Hered. 32, 94.
Africa	Egypt	Nile Delta	32.00	31.50	291.00	0.27	BH	Hussein et al. (1982) Hum Hered. 32, 94.
Africa	Egypt	Suez Canal Zone	32.50	31.00	16.00	0.31	BH	Hussein et al. (1982) Hum Hered. 32, 94.
Africa	Egypt	Upper Egypt, North	28.00	31.00	111.00	0.15	BH	Hussein et al. (1982) Hum Hered. 32, 94.
Africa	Egypt	Upper Egypt, South	28.00	28.00	85.00	0.40	BH	Hussein et al. (1982) Hum Hered. 32, 94.
Africa	Ethiopia	Amhara (phenotyped)	38.66	9.87	94.00	0.34	BH	Jones et al. (2013) Am J Hum Genet. 93, 538.
Africa	Ethiopia	Ethiopians	42.13	9.31	294.00	0.45	BH	Jones et al. (2013) Am J Hum Genet. 93, 538.
Africa	Ethiopia	Oromo (phenotyped)	37.31	7.84	132.00	0.41	BH	Jones et al. (2013) Am J Hum Genet. 93, 538.
Africa	Ethiopia	Somali	41.86	9.59	90.00	0.24	BH	Ingram (2009) J Mol Evol. 69, 579.
Africa	Ethiopia	Somali	41.87	9.58	130.00	0.25	BH	Jones et al. (2013) Am J Hum Genet. 93, 538.
Africa	Ethiopia	Tigray	39.47	13.50	78.00	0.62	BH	Jones et al. (2013) Am J Hum Genet. 93, 538.
Africa	Ethiopia	Wolayta	37.76	6.84	40.00	0.40	BH	Jones et al. (2013) Am J Hum Genet. 93, 538.
Africa	Gabon	Bantu	9.45	0.38	20.00	0.40	BH	Gendrel et al. (1989) J Pediatr Gastroenterol Nutr. 8, 545.
Africa	Kenya	Borana	37.91	-0.02	7.00	0.71	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Kenya	Burji	37.91	-0.02	6.00	0.50	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Kenya	El Molo	37.91	-0.02	6.00	0.67	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Kenya	Gabra	37.91	-0.02	8.00	1.00	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Kenya	Kikuyu	37.91	-0.02	2.00	0.50	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Kenya	Konso	37.91	-0.02	4.00	0.50	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Kenya	Maasai	37.91	-0.02	26.00	0.88	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Kenya	Marakwet	37.91	-0.02	5.00	0.60	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Kenya	Nandi	37.91	-0.02	2.00	0.00	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Kenya	Ogiek	37.91	-0.02	11.00	0.55	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Kenya	Pokot	37.91	-0.02	10.00	0.60	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Kenya	Rendille	37.91	-0.02	7.00	0.71	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Kenya	Sabaot	37.91	-0.02	4.00	0.75	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Kenya	Samburu	37.91	-0.02	9.00	0.89	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Kenya	Sengwer	37.91	-0.02	12.00	0.17	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Kenya	Somali	37.91	-0.02	1.00	1.00	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Kenya	Tugen	37.91	-0.02	11.00	0.73	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Kenya	Turkana	37.91	-0.02	8.00	0.50	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Kenya	Wata	37.91	-0.02	1.00	0.00	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Kenya	Yaaku	37.91	-0.02	11.00	0.73	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.

Africa	Namibia	!Kung	20.50	-19.60	40.00	0.03	BG	Jenkins et al. (1974) Br Med J. 2, 23.
Africa	Namibia	Herero	13.70	-18.30	39.00	0.03	BG	Currie et al. (1978) S Afr J Sci. 74, 227.
Africa	Niger	Tuareg	2.12	13.52	118.00	0.87	BH	Flatz et al. (1986) Am J Hum Genet. 38, 515.
Africa	Nigeria	Hausa/Fulani	3.47	7.23	15.00	0.40	BG	Olatunbosun et al. (1971) Am J Dig Dis. 16, 909.
Africa	Nigeria	Ibo	3.47	7.23	11.00	0.18	BG	Olatunbosun et al. (1971) Am J Dig Dis. 16, 909.
Africa	Nigeria	Yoruba	3.47	7.23	48.00	0.17	BG	Olatunbosun et al. (1971) Am J Dig Dis. 16, 909.
Africa	Rwanda	Hutu-Hutu	29.74	-2.60	36.00	0.58	UG	Cox et al. (1974) Am J Dig Dis. 19, 714.
Africa	Rwanda	Hutu-Tutsi	29.74	-2.60	11.00	0.45	UG	Cox et al. (1974) Am J Dig Dis. 19, 714.
Africa	Rwanda	Shi	29.74	-2.60	28.00	0.04	UG	Cox et al. (1974) Am J Dig Dis. 19, 714.
Africa	Rwanda	Tussi-Tutsi	29.74	-2.60	27.00	0.93	UG	Cox et al. (1974) Am J Dig Dis. 19, 714.
Africa	Senegal	Diolas	-16.25	12.60	40.00	0.73	BG	Arnold et al. (1980) C R Seances Soc Biol Fil. 174, 983.
Africa	Senegal	Peuhls	-15.12	15.40	29.00	1.00	BG	Arnold et al. (1980) C R Seances Soc Biol Fil. 174, 983.
Africa	Senegal	Sereres	-17.43	14.67	38.00	0.71	BG	Arnold et al. (1980) C R Seances Soc Biol Fil. 174, 983.
Africa	Senegal	Toucouleurs	-17.43	14.67	40.00	0.90	BG	Arnold et al. (1980) C R Seances Soc Biol Fil. 174, 983.
Africa	Senegal	Wolof	-17.43	14.67	53.00	0.51	BG	Arnold et al. (1980) C R Seances Soc Biol Fil. 174, 983.
Africa	South Africa	Shangaan	28.08	-26.20	7.00	0.14	BH	Segal et al. (1983) Am J Clin Nutr. 38, 901.
Africa	South Africa	Sotho	28.08	-26.20	23.00	0.35	BH	Segal et al. (1983) Am J Clin Nutr. 38, 901.
Africa	South Africa	Swazi	28.08	-26.20	12.00	0.25	BH	Segal et al. (1983) Am J Clin Nutr. 38, 901.
Africa	South Africa	Tswana	28.08	-26.20	24.00	0.17	BH	Segal et al. (1983) Am J Clin Nutr. 38, 901.
Africa	South Africa	Xhosa	28.08	-26.20	17.00	0.18	BH	Segal et al. (1983) Am J Clin Nutr. 38, 901.
Africa	South Africa	Zulu	31.02	-29.85	47.00	0.11	BG	O'Keefe & Adam (1983) S Afr Med J. 63, 778.
Africa	South Africa	Zulu	28.08	-26.20	32.00	0.19	BH	Segal et al. (1983) Am J Clin Nutr. 38, 901.
Africa	Sudan	Ama	30.22	12.86	2.00	0.50	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Sudan	Amarar	37.22	19.62	82.00	0.87	BH	Bayoumi et al. (1982) Am J Phys Anthropol. 58, 173.
Africa	Sudan	Artega	36.22	19.62	22.00	0.82	BH	Bayoumi et al. (1982) Am J Phys Anthropol. 58, 173.
Africa	Sudan	Bedja	30.95	18.05	9.00	0.89	BH	Bayoumi et al. (1981) Hum Genet. 57, 279.
Africa	Sudan	Beja Banuamir	30.22	12.86	6.00	1.00	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Sudan	Beja Hadandawa	30.22	12.86	11.00	0.82	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Sudan	Beni Amir	37.22	19.62	40.00	0.88	BH	Bayoumi et al. (1982) Am J Phys Anthropol. 58, 173.
Africa	Sudan	Bisharin	37.22	19.62	22.00	0.86	BH	Bayoumi et al. (1982) Am J Phys Anthropol. 58, 173.
Africa	Sudan	Dinka	30.22	12.86	7.00	0.86	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Sudan	Dinka	33.63	7.67	208.00	0.25	BH	Bayoumi et al. (1982) Am J Phys Anthropol. 58, 173.
Africa	Sudan	Dongolawi	30.95	18.05	16.00	0.19	BH	Bayoumi et al. (1981) Hum Genet. 57, 279.
Africa	Sudan	Gomoeia	30.95	18.05	31.00	0.68	BH	Bayoumi et al. (1981) Hum Genet. 57, 279.
Africa	Sudan	Habbani	30.35	13.08	19.00	0.47	BH	Bayoumi et al. (1981) Hum Genet. 57, 279.
Africa	Sudan	Haddendoa	37.22	19.62	137.00	0.80	BH	Bayoumi et al. (1982) Am J Phys Anthropol. 58, 173.
Africa	Sudan	Jaali	32.53	15.59	113.00	0.53	BH	Bayoumi et al. (1981) Hum Genet. 57, 279.
Africa	Sudan	Jaali	33.43	16.69	94.00	0.48	BH	Ingram et al. (2007) Hum Genet. 120, 779.
Africa	Sudan	Kahli	30.95	18.05	21.00	0.62	BH	Bayoumi et al. (1981) Hum Genet. 57, 279.
Africa	Sudan	Koalib	30.22	12.86	1.00	1.00	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Sudan	Liguri/Logorik	30.22	12.86	1.00	0.00	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Sudan	Masalit	30.22	12.86	1.00	1.00	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Sudan	Misseri	30.35	13.08	20.00	0.40	BH	Bayoumi et al. (1981) Hum Genet. 57, 279.

Africa	Sudan	Nilotic	27.67	7.77	18.00	0.33	BH	Bayoumi et al. (1981) Hum Genet. 57, 279.
Africa	Sudan	Nuba	29.68	16.80	58.00	0.21	BH	Bayoumi et al. (1981) Hum Genet. 57, 279.
Africa	Sudan	Nubians	30.95	18.05	21.00	0.33	BH	Bayoumi et al. (1981) Hum Genet. 57, 279.
Africa	Sudan	Nuer	30.22	12.86	2.00	1.00	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Sudan	Nuer	33.63	7.67	23.00	0.22	BH	Bayoumi et al. (1982) Am J Phys Anthropol. 58, 173.
Africa	Sudan	Shaygi	30.95	18.05	42.00	0.38	BH	Bayoumi et al. (1981) Hum Genet. 57, 279.
Africa	Sudan	Shilluk	33.63	7.67	8.00	0.38	BH	Bayoumi et al. (1982) Am J Phys Anthropol. 58, 173.
Africa	Sudan	Shilook	30.22	12.86	4.00	0.75	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Tanzania	Akie	34.89	-6.37	11.00	0.55	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Tanzania	Burunge	34.89	-6.37	16.00	0.38	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Tanzania	Datog	34.89	-6.37	1.00	0.00	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Tanzania	Dorobo	34.89	-6.37	6.00	0.67	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Tanzania	Fiome	34.89	-6.37	7.00	0.14	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Tanzania	Hadza	34.89	-6.37	15.00	0.60	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Tanzania	Iraqw	34.89	-6.37	19.00	0.95	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Tanzania	Maasai	34.89	-6.37	15.00	0.67	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Tanzania	Mbugu	34.89	-6.37	23.00	0.43	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Tanzania	Mbugwe	34.89	-6.37	8.00	0.50	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Tanzania	Pare	34.89	-6.37	8.00	0.75	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Tanzania	Rangi	34.89	-6.37	26.00	0.65	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Tanzania	Samba'a	34.89	-6.37	2.00	0.00	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Tanzania	Sandawe	34.89	-6.37	23.00	0.35	BG	Tishkoff et al. (2007) Nat Genet. 39, 31.
Africa	Tunisia	Tunisian	10.18	36.80	43.00	0.16	BH	Filali et al. (1987) Gastroenterol Clin Biol. 11, 554.
Africa	Uganda	Baganda	32.57	0.32	12.00	0.00	BG	Cook & Dahlquist (1968) Gastroenterol. 55, 328.
Africa	Uganda	Batutsi	32.57	0.32	5.00	1.00	BG	Cook & Dahlquist (1968) Gastroenterol. 55, 328.
Africa	Uganda	Nilotic	32.57	0.32	9.00	0.56	BG	Cook et al. (1966) Lancet. 1, 725.
Africa	Uganda	Ugandan Bantu	32.57	0.32	17.00	0.06	BG	Cook et al. (1966) Lancet. 1, 725.
Africa	Zambia	Bantu of Zambia	28.11	-15.28	26.00	0.00	BG	Cook et al. (1973) Gastroenterol. 64, 405.
Asia	Afghanistan	Hazara	69.18	34.52	10.00	0.20	BG	Rahimi et al. (1976) HumGenet. 34, 57.
Asia	Afghanistan	Mixed urban	69.18	34.52	34.00	0.24	BG	Rahimi et al. (1976) HumGenet. 34, 57.
Asia	Afghanistan	Pasha-I	71.00	36.00	60.00	0.13	BG	Rahimi et al. (1976) HumGenet. 34, 57.
Asia	Afghanistan	Pashtun	69.18	34.52	71.00	0.21	BG	Rahimi et al. (1976) HumGenet. 34, 57.
Asia	Afghanistan	Tajik	69.18	34.52	79.00	0.18	BG	Rahimi et al. (1976) HumGenet. 34, 57.
Asia	Afghanistan	Uzbek	69.18	34.52	16.00	0.00	BG	Rahimi et al. (1976) HumGenet. 34, 57.
Asia	China	Kazakh	87.58	43.80	195.00	0.24	BH	Yongfa et al. (1984) Hum Genet. 67, 103.
Asia	China	Mongols	111.65	40.81	198.00	0.12	BH	Yongfa et al. (1984) Hum Genet. 67, 103.
Asia	China	Northern Han	116.39	39.93	248.00	0.08	BH	Yongfa et al. (1984) Hum Genet. 67, 103.
Asia	India	Indians	72.83	18.98	100.00	0.36	BG	Desai et al. (1970) Indian J Med Sci. 24, 729.
Asia	India	Indians	78.47	17.38	18.00	0.39	BG	Reddy & Pershad (1972) Am J Clin Nutr. 25, 114.
Asia	India	Indians	80.28	13.80	38.00	0.00	Biopsy	Swaminathan et al. (1970) Clin Chim Acta. 30, 707.
Asia	India	Northern Indians	77.20	28.60	70.00	0.73	BG	Gupta et al. (1971) J Trop Med Hyg. 74, 225.
Asia	Japan	Japanese	140.47	40.59	40.00	0.28	BG	Yoshida et al. (1975) Gastroenterol Jpn. 10, 29.
Asia	Myanmar	Burmese	95.96	21.91	50.00	0.08	BG	Aung-Than-Batu et al. (1972) Union Burma J Life Sci. 5, 133.

Asia	Pakistan	Baloochi	67.05	24.87	4.00	1.00	BG	Rab et al. (1976) Br Med J. 1, 436.
Asia	Pakistan	Baluchistani	71.92	32.27	32.00	0.38	BH	Ahmad & Flatz (1984) Hum Hered. 34, 69.
Asia	Pakistan	Kashmiri	71.92	32.27	27.00	0.30	BH	Ahmad & Flatz (1984) Hum Hered. 34, 69.
Asia	Pakistan	Mohajir	67.05	24.87	15.00	0.80	BG	Rab et al. (1976) Br Med J. 1, 436.
Asia	Pakistan	Pathan	67.05	24.87	15.00	1.00	BG	Rab et al. (1976) Br Med J. 1, 436.
Asia	Pakistan	Punjabi	71.92	32.27	322.00	0.41	BH	Ahmad & Flatz (1984) Hum Hered. 34, 69.
Asia	Pakistan	Punjabi	67.05	24.87	9.00	1.00	BG	Rab et al. (1976) Br Med J. 1, 436.
Asia	Pakistan	Punjabi	73.07	33.60	53.00	0.55	BG	Abbas & Ahmad (1983) Hum Genet. 64, 277.
Asia	Pakistan	Sindhi	71.92	32.27	33.00	0.42	BH	Ahmad & Flatz (1984) Hum Hered. 34, 69.
Asia	Pakistan	Sindhi	67.05	24.87	12.00	1.00	BG	Rab et al. (1976) Br Med J. 1, 436.
Asia	Russia	Khanty (Northern)	69.02	61.04	115.00	0.29	BG	Kozlov (1998) Int J Circumpol Heal. 57, 18.
Asia	Russia	Komi-Izhems	50.81	61.67	56.00	0.38	BG	Kozlov (1998) Int J Circumpol Heal. 57, 18.
Asia	Russia	Mansi	66.95	56.96	81.00	0.28	BG	Kozlov (1998) Int J Circumpol Heal. 57, 18.
Asia	Russia	Nenets (West Siberia)	80.86	59.06	9.00	0.22	BG	Kozlov (1998) Int J Circumpol Heal. 57, 18.
Asia	Russia	Udmurtians	53.23	56.85	30.00	0.60	BG	Kozlov (1998) Int J Circumpol Heal. 57, 18.
Asia	Russia	West-Siberian	80.86	59.06	47.00	0.51	BG	Kozlov (1998) Int J Circumpol Heal. 57, 18.
Asia	Sri Lanka	Sri Lankan	80.64	7.30	135.00	0.29	BG	Thomas et al. (1990) J Trop Pediatr. 36, 80.
Asia	Sri Lanka	Sri Lankans ("Ceylonese")	80.60	7.26	200.00	0.28	BG	Senewiratne et al. (1977) Gastroenterol. 72, 1257.
Asia	Taiwan	Chinese	121.45	25.02	50.00	0.12	BG	Sung et al. (1972) Asian J Med. 8, 149.
Asia	Thailand	Thai	100.52	13.75	140.00	0.03	BG	Keusch et al. (1969) Am J Clin Nutr. 22, 638.
Asia	Thailand	Thai	100.49	13.45	40.00	0.00	BG	Troncale et al. (1967) Br Med J. 4, 578.
Australasia	Australia	Aboriginal	123.97	-17.30	45.00	0.16	BH	Brand et al. (1983) Am J Clin Nutr. 37, 449.
Australasia	New Zealand	Maori	174.77	-36.87	28.00	0.36	BH	Abbott & Tasman-Jones (1985) N Z Med J. 10, 228.
Australasia	Papua New Guinea	Central (inc. Port Moresby)	147.19	-9.46	14.00	0.07	BG	Cook (1979) Ann Hum Biol. 6, 55.
Australasia	Papua New Guinea	E and W Sepik provinces	143.52	-4.18	35.00	0.23	BH	Arnhold et al. (1981) Ann Hum Biol. 5, 481
Australasia	Papua New Guinea	Gulf & Western	147.19	-9.46	13.00	0.08	BG	Cook (1979) Ann Hum Biol. 6, 55.
Australasia	Papua New Guinea	Highlands	147.19	-9.46	13.00	0.00	BG	Cook (1979) Ann Hum Biol. 6, 55.
Australasia	Papua New Guinea	Huli, Mendi, and Dunai	142.95	-5.70	30.00	0.10	BG	Jenkins et al. (1981) Ann Hum Biol. 8, 447.
Australasia	Papua New Guinea	Milne Bay	150.60	-10.51	2.00	0.00	BG	Cook (1979) Ann Hum Biol. 6, 55.
Australasia	Papua New Guinea	Morobe & Northern	147.19	-9.46	5.00	0.00	BG	Cook (1979) Ann Hum Biol. 6, 55.
Australasia	Papua New Guinea	N. Solomons & E. New Britain	147.19	-9.46	3.00	0.00	BG	Cook (1979) Ann Hum Biol. 6, 55.
Europe	Austria	Austrian	14.00	47.75	118.00	0.75	BH	Rosenkranz et al. (1982) Hum Genet. 62, 158.
Europe	Austria	Austrian	14.00	47.75	57.00	0.79	BH	Rosenkranz et al. (1982) Hum Genet. 62, 158.
Europe	Austria	Austrian	14.00	47.75	88.00	0.80	BH	Rosenkranz et al. (1982) Hum Genet. 62, 158.
Europe	Austria	Austrian	14.00	47.75	32.00	0.81	BH	Rosenkranz et al. (1982) Hum Genet. 62, 158.
Europe	Austria	Karnten Austrian	14.31	46.62	46.00	0.80	BH	Rosenkranz et al. (1982) Hum Genet. 62, 158.
Europe	Austria	Oberosterreich Austrian	14.30	48.30	45.00	0.84	BH	Rosenkranz et al. (1982) Hum Genet. 62, 158.
Europe	Austria	Tirol Austrian	9.77	47.50	124.00	0.83	BH	Rosenkranz et al. (1982) Hum Genet. 62, 158.
Europe	Cyprus	Greek Cypriots	33.37	35.17	50.00	0.34	BG	Kanaghinis et al. (1974) Am J Dig Dis. 19, 1021.
Europe	Czech Republic	Czech	15.50	49.00	17.00	0.82	BG	Leichter (1972) Am J Dig Dis. 17, 73.
Europe	Denmark	Danes	12.58	55.73	91.00	0.96	BG	Busk et al. (1975) Ugeskr Laeger. 137, 2062.
Europe	Estonia	Estonian	26.40	59.05	112.00	0.75	BG	Lember et al. (1991) Eur J Gastroenterol Hepatol. 3, 479.
Europe	Estonia	Setus	27.64	57.96	100.00	0.51	UG	Lember et al. (1991) Eur J Gastroenterol Hepatol. 3, 479.

Europe	Finland	Finnish-speaking Finns	21.43	60.60	91.00	0.92	BG	Sahi (1974) Scand J Gastroenterol. 9, 303.
Europe	Finland	Finns	27.68	62.90	638.00	0.83	Biopsy	Jussila (1969) Ann Clin Res. 1, 199.
Europe	Finland	Rural Finn	21.93	60.41	159.00	0.83	BG	Jussila et al. (1970) Scand J Gastroenterol. 5, 49.
Europe	Finland	Swedish-speaking Finns	21.43	60.60	156.00	0.83	BG	Sahi (1974) Scand J Gastroenterol. 9, 303.
Europe	France	French	5.82	44.93	102.00	0.76	BH	Cloarec et al. (1991) Gastroenterol Clin Biol. 15, 588.
Europe	France	French	6.63	49.75	85.00	0.71	BH	Cuddenec et al. (1982) Gastroenterol Clin Biol. 6, 776.
Europe	France	Maghrebins (Northern African Muslims)	7.25	43.70	55.00	0.22	BG	O'Morain et al. (1978) Acta Gastroenterol Belg. 41, 56-63.
Europe	France	Northern French	6.63	49.75	76.00	0.78	BH	Cuddenec et al. (1982) Gastroenterol Clin Biol. 6, 776.
Europe	France	Southern French	6.63	49.75	40.00	0.43	BH	Cuddenec et al. (1982) Gastroenterol Clin Biol. 6, 776.
Europe	France	Southern French	7.25	43.70	55.00	0.58	BG	O'Morain et al. (1978) Acta Gastroenterol Belg. 41, 56-63.
Europe	Germany	Baden-Wurttemberg Germans	9.50	48.40	136.00	0.76	BH	Flatz et al. (1982) Hum Genet. 62, 152.
Europe	Germany	Bayern Germans	12.53	47.80	221.00	0.86	BH	Flatz et al. (1982) Hum Genet. 62, 152.
Europe	Germany	Eastern Germans	13.75	51.05	246.00	0.78	BH	Flatz et al. (1982) Hum Genet. 62, 152.
Europe	Germany	Germans	8.52	53.18	60.00	0.87	Biopsy	Howell et al. (1980) Hepatogastroenterology 27, 208.
Europe	Germany	Northwest Germans	8.80	53.08	341.00	0.91	BH	Flatz et al. (1982) Hum Genet. 62, 152.
Europe	Germany	Rheinland and Pfalz Germans	8.27	50.00	182.00	0.86	BH	Flatz et al. (1982) Hum Genet. 62, 152.
Europe	Germany	Schleswig-Holstein Germans	9.55	54.52	100.00	0.94	BH	Flatz et al. (1982) Hum Genet. 62, 152.
Europe	Greece	Continental Greeks	23.73	37.98	600.00	0.55	BG	Kanaghinis et al. (1974) Am J Dig Dis. 19, 1021.
Europe	Greece	Cretan Greek	25.13	35.33	50.00	0.44	BG	Kanaghinis et al. (1974) Am J Dig Dis. 19, 1021.
Europe	Greece	Greek	23.73	37.98	16.00	0.63	BG	Spanidou & Petrakis(1972) Lancet. 2, 872.
Europe	Greece	Greeks	23.73	37.98	200.00	0.25	BH	Ladas et al. (1982) Gut. 23, 968.
Europe	Greece	Greeks	23.73	37.98	250.00	0.77	BG	Zografos et al. (1973) Lancet. 301,367.
Europe	Hungary	Eastern Hungarian	20.08	47.50	70.00	0.71	BH	Czeizel et al. (1983) Hum Genet. 64, 398.
Europe	Hungary	Hungarian	19.08	47.50	262.00	0.59	BH	Czeizel et al. (1983) Hum Genet. 64, 398.
Europe	Hungary	Matyo	20.58	47.82	172.00	0.63	BH	Czeizel et al. (1983) Hum Genet. 64, 398.
Europe	Hungary	Northeastern Hungarian	21.73	47.98	103.00	0.58	BH	Czeizel et al. (1983) Hum Genet. 64, 398.
Europe	Hungary	Romai	21.72	47.95	113.00	0.44	BH	Czeizel et al. (1983) Hum Genet. 64, 398.
Europe	Hungary	Western Hungarian	19.08	47.50	100.00	0.72	BH	Czeizel et al. (1983) Hum Genet. 64, 398.
Europe	Ireland	Native Irish	-6.25	53.33	50.00	0.96	BG	Fielding et al. (1981) Ir J Med Sci. 150, 276.
Europe	Italy	Italians	9.20	45.47	42.00	0.38	BH	Bozzani et al. (1986) Dig Dis Sci. 31, 1313.
Europe	Italy	Italians	9.20	45.47	89.00	0.48	BG	Cavalli-Sforza et al. (1987) Am J Clin Nutr. 45, 748.
Europe	Italy	Italians	12.48	41.90	65.00	0.82	BG	Cavalli-Sforza et al. (1987) Am J Clin Nutr. 45, 748.
Europe	Italy	Italians	14.25	40.83	51.00	0.59	BG	Cavalli-Sforza et al. (1987) Am J Clin Nutr. 45, 748.
Europe	Italy	Italians	14.25	40.83	44.00	0.23	Biopsy	Rossi et al. (1997) Gastroenterology. 112, 1506.
Europe	Italy	Italians	9.20	45.47	20.00	0.25	BH	Zuccato et al. (1983) Eur J Clin Invest. 13, 261.
Europe	Italy	Napolitans	14.25	40.83	99.00	0.46	BH	Rinaldi et al. (1984) Lancet. 1, 355.
Europe	Italy	Neapolitan	14.25	40.83	9.00	0.00	BG	De Ritis et al. (1970) Enzymol Biol Clin. 11, 263.
Europe	Italy	Northern Italians	7.67	45.05	208.00	0.49	BH	Burgio et al. (1984) Am J Clin Nutr. 39, 100.
Europe	Italy	Sardinians	8.56	40.73	50.00	0.14	BH	Meloni et al. (2001) Am J Clin Nutr. 73, 582.
Europe	Italy	Sardinians	9.00	39.40	47.00	0.15	BH	Meloni et al. (1998) Ital J Gastroenterol Hepatol. 30, 490.
Europe	Italy	Sardinians	9.00	40.10	53.00	0.11	BH	Meloni et al. (1998) Ital J Gastroenterol Hepatol. 30, 490.
Europe	Italy	Sardinians	9.00	40.30	38.00	0.18	BH	Meloni et al. (1998) Ital J Gastroenterol Hepatol. 30, 490.
Europe	Italy	Sicilians	13.37	38.12	100.00	0.29	BH	Burgio et al. (1984) Am J Clin Nutr. 39, 100.

Europe	Poland	Eastern Polish	23.13	52.03	35.00	0.63	BH	Socha et al. (1984) Ann Hum Biol. 11, 311.
Europe	Poland	Northeastern Polish	22.35	53.83	34.00	0.59	BH	Socha et al. (1984) Ann Hum Biol. 11, 311.
Europe	Poland	Polish	21.00	52.25	21.00	0.71	BG	Leichter (1972) Am J Dig Dis. 17, 73.
Europe	Poland	Polish	19.00	51.73	29.00	0.62	BH	Socha et al. (1984) Ann Hum Biol. 11, 311.
Europe	Poland	Polish	19.37	52.23	92.00	0.63	BH	Socha et al. (1984) Ann Hum Biol. 11, 311.
Europe	Poland	Polish	19.37	52.23	85.00	0.64	BH	Socha et al. (1984) Ann Hum Biol. 11, 311.
Europe	Russia	Kildin Saami	34.30	69.29	50.00	0.52	BG	Kozlov (1998) Int J Circumpol Heal. 57, 18.
Europe	Russia	Komi-Permiaks	37.59	55.75	112.00	0.50	BG	Kozlov (1998) Int J Circumpol Heal. 57, 18.
Europe	Russia	Udmurtians	32.00	68.00	75.00	0.41	BG	Kozlov (1998) Int J Circumpol Heal. 57, 18.
Europe	Spain	Galician	-7.28	42.37	338.00	0.66	BH	Leis et al. (1997) J Pediatr Gastroenterol Nutr. 25, 296.
Europe	UK	British	-3.20	55.95	150.00	0.95	BG	Ferguson et al. (1984) Gut. 25, 163.
Europe	UK	British natives	-1.25	51.75	75.00	0.95	Biopsy	Ho et al. (1982) Am J Hum Genet. 34, 650.
Europe	UK	White British	-1.92	52.47	67.00	0.97	Biopsy	Iqbal et al. (1993) Br Med J. 306, 1303.
Europe	Uzbekistan	Kazakh	63.46	40.13	83.00	0.25	BG+BH	Heyer et al. (2011) Hum Biol. 83, 379
Europe	Uzbekistan	Tajiko-Uzbek	64.42	39.77	100.00	0.11	BG+BH	Heyer et al. (2011) Hum Biol. 83, 379
Middle East	Iran	Iranian	51.42	35.67	21.00	0.14	BG	Sadre et al. (1979) Am J Clin Nutr. 32, 1948.
Middle East	Israel	Arabs	34.95	32.23	67.00	0.19	BG	Gilat et al. (1971) Dig Dis. 16, 203
Middle East	Jordan	Jordanian Arabs	35.93	31.95	148.00	0.25	BH	Hijazi et al. (1983) Trop Geogr Med. 35, 157.
Middle East	Jordan	Mediterranean origin Jordanian Arabs	35.93	31.95	56.00	0.23	BG	Snook et al. (1976) Trop Geogr Med. 28, 333.
Middle East	Jordan	Urban/agricultural Jordanian Arabs	35.93	31.95	162.00	0.76	BH	Hijazi et al. (1983) Trop Geogr Med. 35, 157.
Middle East	Kuwait	Arab Kuwaiti	47.98	29.37	70.00	0.53	BH	Sanae et al. (2003) Med Princ Pract. 12, 160.
Middle East	Kuwait	Asian Kuwaiti	47.98	29.37	79.00	0.42	BH	Sanae et al. (2003) Med Princ Pract. 12, 160.
Middle East	Lebanon	Lebanese	35.51	33.87	74.00	0.22	BG	Nasrallah (1979) Am J Clin Nutr. 32, 1994.
Middle East	Oman	Omani	58.50	23.53	50.00	0.16	BH	Al-Abri et al. (2013) Oman Med J. 28, 341.
Middle East	Saudi Arabia	Arabs	50.11	26.43	109.00	0.43	BH	Dissanayake et al. (1990) Ann Saudi Med. 10, 598.
Middle East	Saudi Arabia	Bedouin	50.11	26.43	21.00	0.81	BH	Dissanayake et al. (1990) Ann Saudi Med. 10, 598.
Middle East	Saudi Arabia	Beduin and Urban Saudi	46.77	24.64	14.00	0.86	BG	Cook & Al Torki (1975) Br Med J. 3, 135.
Middle East	Saudi Arabia	Yemenites	50.11	26.43	17.00	0.53	BH	Dissanayake et al. (1990) Ann Saudi Med. 10, 598.
Middle East	Turkey	Central Anatolia	39.50	34.00	104.00	0.29	BH	Flatz et al. (1986) Am J Hum Genet. 38, 515.
Middle East	Turkey	Eastern Anatolia	39.50	40.00	122.00	0.26	BH	Flatz et al. (1986) Am J Hum Genet. 38, 515.
Middle East	Turkey	North Coast of Turkey	34.00	41.50	64.00	0.31	BH	Flatz et al. (1986) Am J Hum Genet. 38, 515.
Middle East	Turkey	South Coast of Turkey	33.00	36.50	54.00	0.28	BH	Flatz et al. (1986) Am J Hum Genet. 38, 515.
Middle East	Turkey	Turks	32.86	39.93	30.00	0.63	BG	Tuncbilek et al. (1973) Lancet. 21, 151.
Middle East	Turkey	Western Anatolia and European Turkey	28.96	41.02	126.00	0.30	BH	Flatz et al. (1986) Am J Hum Genet. 38, 515.

Appendix C1: Diversity measures and tests of neutrality for the regions included in haplotype analysis for phenotyped samples. N: number of chromosomes.

Population	N	Sequence region	Segrega- ting sites	Haplo- types	Haplotype diversity, H (\pm SD)	Nucleotide diversity, π	Tajima's D	Fu & Li's D^*	Fu & Li's F^*
Europeans D	40	Intron 4	4	5	0.459 (0.008)	0.001	-1.095	-1.103	-1.282
		Enhancer	3	4	0.500 (0.006)	0.002	0.554	-0.350	-0.096
		Hapdef	2	3	0.349 (0.008)	0.001	-0.406	0.771	0.498
		Total	9	10	0.542 (0.009)	0.001	-0.488	-0.520	-0.598
Europeans ND	30	Intron 4	4	5	0.752 (0.002)	0.003	0.087	0.045	0.066
		Enhancer	2	3	0.480 (0.005)	0.001	0.088	-0.738	-0.583
		Hapdef	3	4	0.690 (0.003)	0.002	0.504	0.950	0.952
		Total	9	9	0.839 (0.001)	0.002	0.298	0.204	0.272
Amhara D	26	Intron 4	2	3	0.665 (0.002)	0.002	1.178	0.826	1.065
		Enhancer	6	7	0.806 (0.003)	0.002	-0.689	0.476	0.157
		Hapdef	2	3	0.665 (0.002)	0.002	1.178	0.826	1.065
		Total	10	8	0.855 (0.002)	0.002	0.263	0.891	0.819
Amhara ND	54	Intron 4	2	3	0.653 (0.001)	0.002	1.832	0.740	1.236
		Enhancer	5	6	0.470 (0.005)	0.001	-1.267	-1.835	-1.940
		Hapdef	2	3	0.662 (0.001)	0.002	1.723	0.740	1.199
		Total	9	10	0.760 (0.002)	0.002	0.430	-0.662	-0.358
Oromo D	50	Intron 4	3	4	0.690 (0.001)	0.003	1.110	0.892	1.115
		Enhancer	8	9	0.722 (0.002)	0.002	-0.898	-1.554	-1.578
		Hapdef	3	4	0.688 (0.000)	0.002	0.953	-0.413	-0.001
		Total	14	12	0.776 (0.002)	0.002	0.079	-0.876	-0.655
Oromo ND	74	Intron 4	3	5	0.559 (0.002)	0.002	0.223	0.853	0.769
		Enhancer	6	7	0.397 (0.005)	0.001	-1.449	-0.690	-1.103
		Hapdef	3	4	0.584 (0.001)	0.001	0.210	-0.515	-0.339
		Total	12	14	0.738 (0.002)	0.001	-0.692	-0.298	-0.516
Jaali D	50	Intron 4	3	4	0.664 (0.001)	0.002	0.477	-0.413	-0.168
		Enhancer	8	9	0.786 (0.001)	0.002	-0.930	-1.554	-1.590
		Hapdef	3	4	0.611 (0.002)	0.002	0.228	-0.413	-0.256
		Total	14	14	0.900 (0.000)	0.002	-0.369	-1.360	-1.213
Jaali ND	26	Intron 4	3	4	0.717 (0.001)	0.003	0.639	-0.216	0.030
		Enhancer	5	6	0.686 (0.008)	0.002	-0.744	0.308	0.003
		Hapdef	3	4	0.717 (0.001)	0.002	0.639	-0.216	0.030
		Total	11	9	0.862 (0.002)	0.002	0.075	-0.001	0.026

Appendix C2: Diversity measures and tests of neutrality for the regions included in haplotype analysis for the combined dataset of 28 populations. N: number of chromosomes, significant values ($p<0.05$) for neutrality tests are shaded.

Population	N	Sequencing region	Segre-gating sites	Haplo-types	Haplotype diversity, H (\pm SD)	Nucleotide diversity, π	Tajima's D	Fu & Li's D^*	Fu & Li's F^*
Afar	124	Intron_4	2	3	0.653 (0.000)	0.002	1.752	0.669	1.180
		Enhancer	7	9	0.754 (0.000)	0.002	0.066	-0.622	-0.459
		Hapdef	2	3	0.644 (0.000)	0.002	1.651	0.669	1.143
		Total	11	12	0.810 (0.000)	0.002	1.108	0.039	0.502
Amhara	80	Intron_4	2	3	0.662 (0.000)	0.002	1.857	0.704	1.231
		Enhancer	7	8	0.600 (0.003)	0.001	-1.154	0.365	-0.165
		Hapdef	2	3	0.661 (0.000)	0.002	1.765	0.704	1.198
		Total	11	12	0.794 (0.001)	0.002	0.291	0.794	0.735
Anatolian Turks	40	Intron_4	4	5	0.694 (0.003)	0.003	0.565	1.027	1.034
		Enhancer	2	3	0.232 (0.007)	0.001	-0.416	0.771	0.495
		Hapdef	3	4	0.679 (0.001)	0.002	0.649	-0.350	-0.063
		Total	9	9	0.771 (0.003)	0.002	0.454	0.733	0.757
Asante	40	Intron_4	3	4	0.714 (0.002)	0.002	0.665	0.916	0.978
		Enhancer	2	3	0.273 (0.008)	0.001	-0.745	0.771	0.384
		Hapdef	2	3	0.472 (0.006)	0.001	0.171	0.771	0.692
		Total	7	7	0.833 (0.001)	0.001	0.122	1.256	1.056
Beni Amer	128	Intron_4	5	5	0.654 (0.000)	0.002	-0.198	-2.266	-1.867
		Enhancer	5	6	0.786 (0.000)	0.002	1.026	1.016	1.205
		Hapdef	3	4	0.612 (0.001)	0.002	0.591	0.807	0.868
		Total	13	14	0.855 (0.000)	0.002	0.614	-0.364	-0.008
Chagga	82	Intron_4	3	4	0.703 (0.001)	0.003	1.014	0.844	1.050
		Enhancer	4	5	0.459 (0.004)	0.001	-0.753	-0.220	-0.457
		Hapdef	2	3	0.417 (0.003)	0.001	0.185	0.702	0.635
		Total	9	9	0.863 (0.000)	0.001	0.114	0.603	0.517
Chewa	40	Intron_4	5	5	0.733 (0.001)	0.003	-0.266	-0.736	-0.692
		Enhancer	3	4	0.273 (0.008)	0.001	-1.177	-1.616	-1.727
		Hapdef	2	3	0.512 (0.002)	0.001	0.243	-0.828	-0.599
		Total	10	10	0.829 (0.001)	0.001	-0.562	-1.506	-1.415
Iranians	76	Intron_4	4	5	0.675 (0.001)	0.003	0.628	0.965	1.007
		Enhancer	2	3	0.362 (0.003)	0.001	-0.017	0.708	0.569
		Hapdef	3	4	0.674 (0.000)	0.002	1.038	-0.521	-0.044
		Total	9	8	0.726 (0.001)	0.002	0.819	0.617	0.811
Israeli-Arabs	40	Intron_4	6	6	0.736 (0.002)	0.003	-0.457	-0.456	-0.533
		Enhancer	7	8	0.555 (0.007)	0.001	-1.632	-2.464	-2.5812
		Hapdef	2	3	0.656 (0.001)	0.002	1.407	0.771	1.107
		Total	15	13	0.837 (0.002)	0.002	-0.778	-1.460	-1.457
Israeli-Bedouin	32	Intron_4	3	4	0.720 (0.001)	0.003	0.860	0.942	1.064
		Enhancer	5	6	0.641 (0.006)	0.002	-0.836	-1.526	-1.537
		Hapdef	2	3	0.677 (0.001)	0.002	1.515	0.798	1.158
		Total	10	10	0.861 (0.001)	0.002	0.333	-0.240	-0.072
Italians	32	Intron_4	4	5	0.752 (0.001)	0.003	0.171	1.051	0.920
		Enhancer	3	4	0.504 (0.006)	0.001	-0.478	-1.508	-1.402
		Hapdef	3	4	0.702 (0.002)	0.002	0.641	0.942	0.991
		Total	10	12	0.867 (0.001)	0.002	0.151	0.309	0.304
Jaali	76	Intron_4	3	4	0.682 (0.000)	0.002	0.825	0.851	0.986
		Enhancer	8	9	0.752 (0.001)	0.002	-0.829	-1.039	-1.144
		Hapdef	3	4	0.654 (0.001)	0.002	0.692	0.851	0.938
		Total	14	14	0.891 (0.000)	0.002	-0.042	-0.057	-0.061
Jordanian Bedouin	44	Intron_4	2	3	0.672 (0.001)	0.003	1.919	0.761	1.275
		Enhancer	4	5	0.725 (0.002)	0.002	0.781	1.017	1.103
		Hapdef	3	4	0.707 (0.001)	0.002	1.170	0.905	1.145
		Total	9	7	0.775 (0.002)	0.002	1.616	1.357	1.692
Khalka	38	Intron_4	3	4	0.585 (0.005)	0.002	-0.110	0.922	0.718
		Enhancer	3	4	0.542 (0.003)	0.001	-0.334	-1.592	-1.419
		Hapdef	2	3	0.519 (0.006)	0.001	0.403	0.777	0.775
		Total	8	8	0.795 (0.001)	0.001	-0.066	-0.030	-0.048
Kuwaiti	56	Intron_4	3	5	0.647 (0.002)	0.003	0.992	-0.444	-0.008
		Enhancer	6	7	0.639 (0.003)	0.002	-0.867	-1.464	-1.495
		Hapdef	3	4	0.662 (0.001)	0.002	0.940	-0.444	-0.027
		Total	12	12	0.822 (0.001)	0.002	0.190	-1.300	-0.942

Mambila	40	Intron_4	3	4	0.509 (0.006)	0.002	-0.425	0.916	0.603
		Enhancer	3	4	0.355 (0.009)	0.001	-0.745	0.916	0.493
		Hapdef	1	2	0.328 (0.006)	0.001	0.565	0.564	0.651
		Total	7	7	0.731 (0.004)	0.001	-0.480	1.256	0.836
Northern Europeans	40	Intron_4	3	4	0.458 (0.008)	0.001	-0.615	0.916	0.538
		Enhancer	3	4	0.522 (0.005)	0.002	0.681	-0.350	-0.052
		Hapdef	2	3	0.349 (0.008)	0.001	-0.406	0.771	0.498
		Total	8	8	0.569 (0.008)	0.001	-0.121	0.631	0.463
Norwegians	40	Intron_4	2	3	0.229 (0.007)	0.001	-0.946	-0.828	-0.999
		Enhancer	2	3	0.268 (0.007)	0.001	0.063	0.771	0.656
		Hapdef	2	3	0.229 (0.007)	0.001	-0.946	-0.828	-0.999
		Total	6	4	0.273 (0.008)	0.001	-0.862	-0.456	-0.680
Oromo	124	Intron_4	3	5	0.643 (0.001)	0.002	1.041	0.809	1.039
		Enhancer	9	10	0.598 (0.002)	0.001	-1.214	0.529	-0.097
		Hapdef	4	5	0.655 (0.000)	0.002	0.295	-1.545	-1.116
		Total	16	18	0.798 (0.001)	0.002	-0.355	-0.005	-0.162
Palestinians	38	Intron_4	6	6	0.758 (0.001)	0.003	-0.293	-1.250	-1.118
		Enhancer	3	4	0.422 (0.007)	0.001	-0.735	-1.592	-1.555
		Hapdef	3	4	0.684 (0.001)	0.002	0.533	-0.335	-0.093
		Total	12	13	0.862 (0.001)	0.002	-0.240	-1.541	-1.321
Romanians	40	Intron_4	4	5	0.713 (0.001)	0.003	0.047	-1.103	-0.882
		Enhancer	2	3	0.344 (0.007)	0.001	0.480	0.771	0.796
		Hapdef	3	4	0.704 (0.002)	0.002	0.887	0.916	1.054
		Total	9	9	0.859 (0.001)	0.002	0.587	0.107	0.304
Saudi Bedouin	40	Intron_4	2	3	0.640 (0.002)	0.002	1.814	0.771	1.244
		Enhancer	5	6	0.674 (0.002)	0.002	-0.555	-1.662	-1.546
		Hapdef	2	3	0.653 (0.001)	0.002	1.711	0.771	1.209
		Total	9	10	0.773 (0.003)	0.002	0.858	-0.520	-0.097
Shabo	42	Intron_4	5	6	0.791 (0.001)	0.003	0.042	0.178	0.159
		Enhancer	3	4	0.297 (0.007)	0.001	-1.196	-1.639	-1.752
		Hapdef	2	3	0.487 (0.003)	0.001	0.153	-0.843	-0.641
		Total	10	11	0.854 (0.001)	0.001	-0.419	-0.953	-0.919
Syrians	82	Intron_4	3	4	0.656 (0.001)	0.003	1.035	0.844	1.058
		Enhancer	2	3	0.427 (0.003)	0.001	0.171	0.702	0.630
		Hapdef	4	5	0.680 (0.001)	0.002	0.371	-0.220	-0.038
		Total	9	11	0.736 (0.001)	0.002	0.744	0.603	0.771
Tharu	36	Intron_4	4	5	0.668 (0.003)	0.002	-0.362	-0.008	-0.132
		Enhancer	2	3	0.341 (0.009)	0.001	-0.012	0.783	0.642
		Hapdef	2	3	0.595 (0.003)	0.001	0.807	0.783	0.914
		Total	8	8	0.825 (0.001)	0.001	0.083	0.653	0.557
Ukrainians	40	Intron_4	3	4	0.664 (0.002)	0.002	0.344	0.916	0.868
		Enhancer	3	4	0.627 (0.003)	0.002	0.887	-0.350	0.018
		Hapdef	3	4	0.558 (0.004)	0.001	-0.195	-0.350	-0.353
		Total	9	7	0.795 (0.001)	0.002	0.463	0.107	0.258
Yemeni Hadramaut	154	Intron_4	4	5	0.660 (0.000)	0.003	0.587	-0.356	-0.055
		Enhancer	9	10	0.675 (0.001)	0.002	-1.041	-1.907	-1.906
		Hapdef	4	5	0.680 (0.000)	0.002	0.645	0.905	0.969
		Total	17	20	0.867 (0.000)	0.002	-0.209	-1.056	-0.878
Yemeni Sena	66	Intron_4	4	5	0.716 (0.001)	0.003	0.331	0.978	0.907
		Enhancer	6	7	0.770 (0.001)	0.002	0.195	1.153	0.992
		Hapdef	2	3	0.610 (0.001)	0.002	1.163	0.721	0.994
		Total	12	13	0.892 (0.000)	0.002	0.587	1.476	1.387

Appendix D: Pairwise F_{ST} values (lower triangle) and corresponding p -values (upper triangles) for the 52 populations tested in chapter 3.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1	*	0.045	0.559	0.036	0.000	0.000	0.000	0.270	0.000	0.991	0.000	0.000	0.135	0.297	0.117	0.000	0.009	0.099	0.000	0.000	0.000	0.459	0.000	0.000	0.459
2	0.035	*	0.261	0.000	0.387	0.225	0.000	0.793	0.000	0.090	0.000	0.000	0.739	0.000	0.000	0.000	0.000	0.000	0.000	0.396	0.009	0.018	0.000	0.784	0.721
3	-0.009	0.002	*	0.000	0.171	0.036	0.000	0.676	0.000	0.550	0.000	0.000	0.550	0.216	0.027	0.000	0.009	0.027	0.000	0.090	0.000	0.225	0.027	0.153	0.991
4	0.033	0.124	0.072	*	0.000	0.000	0.000	0.009	0.000	0.072	0.000	0.991	0.000	0.432	0.586	0.000	0.955	0.559	0.000	0.000	0.000	0.477	0.000	0.000	0.135
5	0.077	-0.001	0.029	0.169	*	0.748	0.000	0.234	0.000	0.018	0.000	0.000	0.169	0.000	0.000	0.000	0.000	0.000	0.000	0.991	0.099	0.000	0.153	0.586	0.333
6	0.117	0.007	0.048	0.245	-0.011	*	0.000	0.342	0.000	0.009	0.000	0.000	0.144	0.000	0.000	0.000	0.000	0.000	0.865	0.117	0.009	0.468	0.396	0.333	0.333
7	0.625	0.479	0.528	0.692	0.401	0.367	*	0.000	0.342	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
8	0.008	-0.013	-0.017	0.129	0.006	0.019	0.498	*	0.000	0.387	0.000	0.000	0.991	0.045	0.009	0.000	0.000	0.018	0.000	0.351	0.045	0.171	0.072	0.514	0.991
9	0.727	0.570	0.632	0.813	0.484	0.459	-0.003	0.605	*	0.000	0.009	0.000	0.000	0.000	0.000	0.036	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
10	-0.009	0.030	-0.011	0.043	0.070	0.110	0.621	0.004	0.727	*	0.000	0.018	0.234	0.234	0.045	0.000	0.018	0.072	0.000	0.009	0.000	0.360	0.000	0.027	0.730
11	0.452	0.266	0.320	0.554	0.185	0.147	0.068	0.283	0.129	0.446	*	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.270	0.000	0.045	0.000
12	0.049	0.158	0.111	-0.010	0.208	0.307	0.736	0.180	0.851	0.063	0.621	*	0.009	0.135	0.225	0.000	0.631	0.405	0.000	0.000	0.000	0.189	0.000	0.000	0.054
13	0.015	-0.006	-0.007	0.085	0.010	0.024	0.507	-0.016	0.592	0.011	0.301	0.108	*	0.018	0.009	0.000	0.000	0.000	0.144	0.000	0.000	0.063	0.009	0.441	0.991
14	0.001	0.074	0.016	0.004	0.118	0.170	0.651	0.049	0.755	0.007	0.492	0.014	0.045	*	0.865	0.000	0.162	0.757	0.000	0.000	0.000	0.856	0.000	0.000	0.144
15	0.011	0.102	0.033	-0.002	0.156	0.224	0.703	0.078	0.801	0.015	0.565	0.002	0.066	-0.005	*	0.000	0.081	0.991	0.000	0.000	0.000	0.991	0.000	0.000	0.099
16	0.863	0.742	0.835	0.944	0.668	0.681	0.129	0.816	0.087	0.867	0.363	0.956	0.749	0.888	0.906	*	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
17	0.048	0.139	0.084	-0.017	0.183	0.256	0.695	0.140	0.808	0.057	0.558	-0.005	0.099	0.016	0.010	0.934	*	0.153	0.000	0.000	0.000	0.108	0.000	0.000	0.036
18	0.016	0.107	0.043	-0.005	0.156	0.226	0.694	0.091	0.802	0.021	0.554	-0.003	0.070	-0.006	-0.007	0.920	0.007	*	0.000	0.000	0.000	0.991	0.000	0.000	0.081
19	0.183	0.177	0.130	0.197	0.187	0.192	0.556	0.143	0.607	0.186	0.386	0.242	0.169	0.180	0.216	0.749	0.197	0.211	*	0.000	0.000	0.000	0.000	0.000	0.018
20	0.088	0.002	0.037	0.186	-0.010	-0.013	0.394	0.011	0.479	0.082	0.178	0.228	0.016	0.133	0.173	0.667	0.200	0.173	0.195	*	0.090	0.000	0.243	0.468	0.514
21	0.310	0.115	0.172	0.464	0.051	0.026	0.195	0.135	0.279	0.302	0.015	0.548	0.146	0.363	0.449	0.558	0.464	0.447	0.265	0.046	*	0.000	0.532	0.027	0.081
22	-0.002	0.067	0.012	-0.001	0.106	0.154	0.628	0.047	0.747	0.002	0.457	0.013	0.040	-0.011	-0.012	0.912	0.015	-0.011	0.155	0.120	0.334	*	0.000	0.027	0.216
23	0.221	0.058	0.111	0.369	0.014	-0.004	0.264	0.076	0.350	0.214	0.061	0.449	0.085	0.275	0.353	0.610	0.374	0.352	0.225	0.009	-0.018	0.250	*	0.018	0.198
24	0.052	-0.007	0.014	0.139	-0.006	-0.003	0.450	-0.005	0.536	0.047	0.235	0.172	0.000	0.093	0.123	0.706	0.154	0.125	0.183	-0.005	0.089	0.084	0.038	*	0.541
25	-0.006	-0.022	-0.030	0.156	-0.003	0.011	0.489	-0.036	0.601	-0.010	0.265	0.227	-0.026	0.038	0.069	0.843	0.157	0.090	0.121	0.003	0.116	0.042	0.063	-0.014	*
26	0.745	0.608	0.671	0.817	0.530	0.513	0.016	0.646	-0.011	0.745	0.179	0.848	0.627	0.770	0.808	0.053	0.814	0.808	0.651	0.526	0.345	0.764	0.414	0.576	0.648
27	0.598	0.427	0.479	0.683	0.342	0.304	-0.005	0.446	0.021	0.594	0.026	0.736	0.458	0.629	0.690	0.203	0.684	0.682	0.506	0.335	0.131	0.602	0.196	0.395	0.432
28	0.045	-0.021	-0.002	0.210	-0.016	-0.010	0.442	-0.021	0.548	0.039	0.216	0.281	-0.015	0.100	0.146	0.788	0.211	0.162	0.146	-0.013	0.074	0.098	0.028	-0.020	-0.030
29	0.163	0.020	0.072	0.327	-0.009	-0.020	0.329	0.038	0.425	0.156	0.109	0.406	0.042	0.224	0.292	0.682	0.332	0.299	0.200	-0.012	0.001	0.208	-0.019	0.006	0.029
30	0.003	0.040	-0.001	0.028	0.073	0.099	0.572	0.015	0.665	0.004	0.383	0.046	0.023	0.008	0.011	0.830	0.038	0.019	0.087	0.083	0.240	0.002	0.173	0.055	0.001
31	-0.019	0.030	-0.016	0.025	0.064	0.096	0.577	0.006	0.693	-0.018	0.381	0.054	0.011	-0.014	-0.009	0.892	0.039	-0.003	0.127	0.075	0.242	-0.019	0.171	0.045	-0.007
32	0.294	0.141	0.195	0.385	0.081	0.053	0.167	0.158	0.237	0.286	0.012	0.442	0.171	0.335	0.397	0.447	0.396	0.386	0.286	0.074	-0.019	0.306	0.001	0.116	0.147
33	-0.011	0.043	-0.006	0.023	0.080	0.122	0.611	0.018	0.725	-0.010	0.433	0.042	0.019	-0.006	-0.009	0.887	0.029	-0.003	0.125	0.094	0.295	-0.012	0.216	0.059	0.006
34	0.028	-0.009	0.000	0.107	0.002	0.011	0.485	-0.014	0.572	0.024	0.275	0.135	-0.006	0.064	0.089	0.735	0.122	0.092	0.175	0.006	0.123	0.057	0.065	-0.005	-0.024
35	0.099	-0.003	0.034	0.244	-0.016	-0.019	0.388	0.007	0.485	0.093	0.165	0.311	0.011	0.155	0.209	0.718	0.253	0.217	0.178	-0.016	0.037	0.143	0.002	-0.011	-0.002
36	0.158	0.031	0.077	0.276	-0.001	-0.011	0.313	0.046	0.397	0.150	0.101	0.338	0.051	0.204	0.266	0.621	0.287	0.264	0.209	-0.002	-0.002	0.186	-0.015	0.017	0.036
37	0.391	0.210	0.265	0.495	0.136	0.101	0.110	0.227	0.177	0.383	-0.011	0.562	0.244	0.433	0.506	0.412	0.501	0.495	0.348	0.128	-0.005	0.398	0.028	0.181	0.211
38	0.001	0.006	-0.012	0.059	0.031	0.050	0.539	-0.011	0.627	0.000	0.341	0.081	-0.002	0.026	0.041	0.783	0.073	0.046	0.159	0.038	0.189	0.020	0.121	0.016	-0.024
39	0.280	0.113	0.169	0.397	0.055	0.030	0.204	0.132	0.282	0.272	0.026	0.465	0.143	0.329	0.400	0.520	0.405	0.392	0.278	0.049	-0.022	0.298	-0.013	0.088	0.120
40	0.687	0.530	0.590	0.768	0.445	0.417	-0.010	0.562	-0.011	0.686	0.094	0.809	0.554	0.716	0.764	0.127	0.766	0.762	0.584	0.440	0.237	0.701	0.309	0.496	0.557
41	0.023	0.110	0.043	0.003	0.160	0.221	0.690	0.086	0.785	0.029	0.548	0.009	0.076	0.004	0.000	0.894	0.006	0.002	0.168	0.176	0.429	-0.001	0.339	0.130	0.077
42	0.115	-0.002	0.036	0.286	-0.021	-0.024	0.363	0.008	0.459	0.108	0.138	0.368	0.011	0.175	0.238	0.719	0.286	0.247	0.166	-0.020	0.014	0.162	-0.010	-0.011	-0.002
43	0.035	-0.011	0.001	0.133	-0.002	0.006	0.475	-0.015	0.570	0.029	0.261	0.173	-0.009	0.076	0.107	0.755	0.147	0.113	0.168	0.001	0.112	0.069	0.056	-0.008	-0.024
44	0.016	-0.008	-0.009	0.100	0.008	0.021	0.500	-0.018	0.594	0.011	0.290	0.132	-0.010	0.048	0.074	0.771	0.114	0.080	0.160	0.014	0.139	0.045	0.079	-0.002	-0.029
45	0.289	0.128	0.183	0.392	0.069	0.042	0.185	0.146	0.259	0.281	0.018	0.455	0.158	0.334	0.401	0.483	0.402	0.391	0.291	0.062	-0.020	0.303	-0.006	0.103	0.134
46	0.210	0.075	0.128	0.305	0.031	0.011	0.246	0.093	0.321	0.202	0.056	0.356	0.101	0.254	0.309	0.525	0.317	0.301	0.257	0.025	-0.016	0.230	-0.016	0.056	0.084
47	0.106	0.008	0.045	0.212	-0.010	-0.015	0.365	0.018	0.449	0.098	0.148	0.263	0.022	0.151	0.200	0.654	0.225	0.199	0.189						

	26	27	28	29	30	31	32	33	34	35	36	37	38	39	40	41	42	43	44	45	46	47	48	49	50	51
1	0.000	0.000	0.090	0.000	0.270	0.991	0.000	0.757	0.045	0.018	0.000	0.000	0.225	0.000	0.000	0.027	0.018	0.045	0.126	0.000	0.000	0.000	0.369	0.000	0.703	0.000
2	0.000	0.000	0.991	0.216	0.027	0.216	0.000	0.117	0.991	0.532	0.045	0.000	0.225	0.009	0.000	0.000	0.324	0.991	0.541	0.000	0.027	0.198	0.027	0.315	0.027	0.000
3	0.000	0.000	0.077	0.054	0.441	0.649	0.009	0.342	0.387	0.144	0.009	0.000	0.694	0.000	0.000	0.045	0.090	0.279	0.432	0.000	0.000	0.072	0.108	0.036	0.297	0.000
4	0.000	0.000	0.409	0.000	0.045	0.189	0.000	0.171	0.000	0.000	0.000	0.000	0.036	0.000	0.000	0.288	0.000	0.000	0.000	0.000	0.000	0.000	0.360	0.000	0.396	0.000
5	0.000	0.000	0.658	0.559	0.000	0.036	0.000	0.000	0.333	0.991	0.342	0.000	0.081	0.072	0.000	0.000	0.883	0.441	0.225	0.009	0.045	0.811	0.000	0.991	0.009	0.000
6	0.000	0.000	0.649	0.802	0.018	0.054	0.045	0.000	0.279	0.991	0.622	0.000	0.036	0.162	0.000	0.000	0.892	0.198	0.162	0.081	0.297	0.991	0.000	0.991	0.000	0.000
7	0.108	0.514	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.739	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
8	0.000	0.000	0.838	0.135	0.252	0.505	0.000	0.243	0.874	0.324	0.099	0.000	0.811	0.009	0.000	0.018	0.315	0.811	0.991	0.000	0.018	0.144	0.099	0.279	0.135	0.000
9	0.667	0.162	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.568	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
10	0.000	0.000	0.099	0.000	0.378	0.712	0.000	0.658	0.126	0.027	0.000	0.000	0.243	0.000	0.000	0.027	0.009	0.054	0.162	0.000	0.000	0.000	0.333	0.009	0.468	0.000
11	0.000	0.153	0.000	0.000	0.000	0.000	0.306	0.000	0.000	0.000	0.036	0.676	0.000	0.162	0.018	0.000	0.009	0.000	0.000	0.189	0.027	0.000	0.000	0.000	0.000	0.000
12	0.000	0.000	0.000	0.000	0.000	0.090	0.000	0.045	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.153	0.000	0.000	0.000	0.000	0.000	0.279	0.000	0.171	0.000	0.000
13	0.000	0.000	0.766	0.081	0.099	0.234	0.000	0.162	0.757	0.252	0.027	0.000	0.423	0.000	0.000	0.000	0.279	0.793	0.991	0.000	0.000	0.117	0.018	0.162	0.045	0.000
14	0.000	0.000	0.054	0.000	0.270	0.676	0.000	0.631	0.000	0.000	0.000	0.000	0.081	0.000	0.000	0.288	0.000	0.000	0.027	0.000	0.000	0.000	0.892	0.000	0.559	0.000
15	0.000	0.000	0.018	0.000	0.189	0.685	0.000	0.775	0.000	0.000	0.000	0.000	0.009	0.000	0.000	0.369	0.000	0.000	0.000	0.000	0.000	0.000	0.991	0.000	0.586	0.000
16	0.018	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
17	0.000	0.000	0.000	0.000	0.000	0.081	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.171	0.000	0.000	0.000	0.000	0.000	0.000	0.126	0.000	0.207	0.000
18	0.000	0.000	0.000	0.000	0.090	0.595	0.000	0.387	0.000	0.000	0.000	0.000	0.036	0.000	0.000	0.207	0.000	0.000	0.000	0.000	0.000	0.000	0.847	0.000	0.459	0.000
19	0.000	0.000	0.000	0.000	0.018	0.018	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.793
20	0.000	0.000	0.640	0.649	0.000	0.054	0.018	0.000	0.324	0.991	0.441	0.000	0.009	0.027	0.000	0.000	0.820	0.387	0.198	0.018	0.126	0.739	0.000	0.991	0.000	0.000
21	0.000	0.000	0.072	0.252	0.000	0.000	0.829	0.000	0.000	0.153	0.405	0.306	0.000	0.838	0.000	0.000	0.315	0.009	0.000	0.865	0.604	0.126	0.000	0.162	0.000	0.000
22	0.000	0.000	0.036	0.000	0.523	0.991	0.000	0.739	0.000	0.000	0.000	0.000	0.162	0.000	0.000	0.450	0.000	0.027	0.108	0.000	0.000	0.000	0.991	0.009	0.766	0.000
23	0.000	0.000	0.171	0.784	0.000	0.009	0.351	0.000	0.045	0.324	0.541	0.090	0.009	0.586	0.000	0.000	0.468	0.009	0.009	0.432	0.739	0.351	0.000	0.297	0.000	0.000
24	0.000	0.000	0.991	0.333	0.009	0.153	0.000	0.063	0.649	0.685	0.099	0.000	0.099	0.000	0.000	0.000	0.459	0.667	0.450	0.000	0.045	0.396	0.009	0.568	0.000	0.000
25	0.000	0.000	0.775	0.315	0.432	0.685	0.036	0.450	0.811	0.351	0.081	0.009	0.910	0.045	0.000	0.000	0.550	0.757	0.991	0.000	0.135	0.279	0.261	0.486	0.586	0.000
26	*	0.036	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.207	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
27	0.050	*	0.000	0.000	0.000	0.000	0.009	0.000	0.000	0.000	0.000	0.063	0.000	0.000	0.315	0.000	0.000	0.000	0.000	0.009	0.000	0.000	0.000	0.000	0.000	0.000
28	0.599	0.382	*	0.405	0.081	0.333	0.054	0.099	0.991	0.757	0.261	0.000	0.450	0.036	0.000	0.000	0.649	0.991	0.730	0.045	0.108	0.477	0.009	0.703	0.063	0.000
29	0.485	0.262	-0.001	*	0.000	0.018	0.117	0.000	0.153	0.649	0.775	0.036	0.036	0.324	0.000	0.000	0.721	0.144	0.036	0.216	0.468	0.829	0.000	0.640	0.000	0.000
30	0.697	0.530	0.040	0.129	*	0.838	0.000	0.910	0.009	0.027	0.000	0.000	0.270	0.000	0.000	0.297	0.000	0.018	0.108	0.000	0.000	0.000	0.333	0.000	0.324	0.000
31	0.722	0.537	0.039	0.132	-0.011	*	0.000	0.991	0.180	0.144	0.009	0.000	0.505	0.000	0.000	0.225	0.081	0.090	0.324	0.000	0.000	0.009	0.649	0.027	0.991	0.009
32	0.290	0.110	0.105	0.027	0.249	0.246	*	0.000	0.000	0.018	0.099	0.541	0.000	0.640	0.000	0.000	0.108	0.000	0.000	0.856	0.396	0.036	0.000	0.027	0.000	0.000
33	0.745	0.582	0.058	0.168	-0.019	-0.024	0.282	*	0.036	0.009	0.000	0.000	0.297	0.000	0.000	0.514	0.000	0.072	0.144	0.000	0.000	0.000	0.694	0.000	0.991	0.000
34	0.609	0.434	-0.019	0.026	0.035	0.023	0.149	0.035	*	0.486	0.054	0.000	0.234	0.000	0.000	0.000	0.342	0.991	0.838	0.000	0.009	0.207	0.018	0.252	0.027	0.000
35	0.539	0.325	-0.019	-0.018	0.083	0.082	0.065	0.107	0.000	*	0.523	0.000	0.117	0.117	0.000	0.000	0.856	0.577	0.288	0.054	0.252	0.991	0.000	0.991	0.000	0.000
36	0.454	0.248	0.009	-0.020	0.131	0.125	0.025	0.151	0.037	-0.009	*	0.027	0.009	0.189	0.000	0.000	0.829	0.072	0.027	0.090	0.414	0.622	0.000	0.378	0.000	0.000
37	0.231	0.059	0.164	0.067	0.329	0.324	-0.005	0.373	0.219	0.117	0.064	*	0.000	0.306	0.000	0.000	0.018	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
38	0.660	0.494	0.000	0.074	0.011	-0.004	0.206	0.006	0.004	0.035	0.083	0.284	*	0.000	0.000	0.000	0.225	0.333	0.441	0.000	0.000	0.018	0.108	0.081	0.099	0.000
39	0.341	0.142	0.078	0.007	0.232	0.227	-0.011	0.270	0.121	0.041	0.008	0.003	0.182	*	0.000	0.000	0.162	0.000	0.009	0.820	0.595	0.108	0.000	0.072	0.000	0.000
40	0.004	0.002	0.504	0.382	0.628	0.648	0.199	0.681	0.533	0.443	0.358	0.139	0.590	0.242	*	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000	0.000
41	0.795	0.674	0.147	0.282	0.006	0.004	0.386	-0.004	0.097	0.207	0.262	0.491	0.047	0.388	0.749	*	0.000	0.000	0.000	0.000	0.000	0.000	0.342	0.000	0.207	0.000
42	0.521	0.295	-0.020	-0.025	0.088	0.089	0.048	0.122	0.002	-0.026	-0.022	0.094	0.039	0.025	0.418	0.232	*	0.270	0.234	0.126	0.225	0.955	0.000	0.820	0.000	0.000
43	0.610	0.423	-0.022	0.019	0.038	0.029	0.137	0.043	-0.010	-0.004	0.030	0.205	0.004	0.110	0.529	0.114	-0.004	*	0.847	0.000	0.027	0.189	0.000	0.333	0.009	0.000
44	0.632	0.450	-0.017	0.039	0.023	0.011	0.163	0.019	-0.008	0.008	0.046	0.234	-0.004	0.136	0.554	0.083	0.008	-0.010	*	0.000	0.009	0.117	0.063	0.243	0.036	0.000
45	0.315	0.125	0.092	0.017	0.244	0.238	-0.011	0.277	0.136	0.053	0.017	-0.002	0.196	-0.014	0.219	0.391	0.037	0.124	0.151	*	0.514	0.045	0.000	0.045	0.000	0.000
46	0.374	0.184	0.048	-0.006	0.																					

Appendix E: Summary of the functional studies conducted during this thesis.
Abbreviations: Comp: Competitor, AB: Antibody. +: has an effect, bold crosses: strong effect, - no effect, (+) weak effect in competitions or slight inhibition in supershift.

		-14028*T	-14028*C	-14011*C	-14011*T	-14009*G	-13779*G	-13779*C
Transfections								
	2 days		+		+	+		+
	9 days		-		+	+		-
EMSAs								
Cdx-2	Comp	+	+	+	+	+	(+)	(+)
	AB	+	-	(+)	(+)	(+)	-	-
Oct-1	Comp	+	(+)	+	+	+	(+)	-
	AB	-	-	+	+	+		
HNF-1α	Comp	+	+	+	+	+	(+)	-
	AB	-	-	(+)	(+)	(+)		
HNF-4α	Comp	-	+				+	+
	AB	-	+				+	+
GATA 3, 4	Comp			+	+			
c-Ets-1,	Comp			-		+		
Aff_ELF	Comp			+		-		
Aff_ELK-1	Comp			+		+		
Aff_ETS (1)	Comp			(+)		+		
Ets/Tel2, Aff_c-Ets-1	Comp			-		-		
Ets-1, Ets 1/2	AB			-		-		
NF_kappaB, Pax,								
Pax4-8, Pbx	Comp			(+)		(+)		
LEF-1,	Comp						-	-
GKLF-1, AML-1	Comp						(+)	(+)
Bioinf. Pred.								
TRANSFAC		CdxA	HNF4a	Oct, CdxA	Oct, CdxA, GATA4	Oct, CdxA, c-Ets-1, Ets	-	HNF4
MatInspector		Cdx2, others		Oct1, HNF1	GATA3, Oct1, HNF1	ELF5, Oct1, HNFP	-	RU49, LEF1, GKLF
TFSEARCH		CdxA, others	HNF4a	Oct1, CdxA, others	Oct1, CdxA, others	ELK1, ELK4, c-Ets, Oct1, CdxA, others	-	AML-1a

Appendix F: Pairwise linkage disequilibrium D' across the 80kb haplotype region of the Middle Eastern groups tested. Monomorphic markers were excluded from analysis. Statistically significant values after Bonferroni correction for multiple testing for the respective amount of populations per group are shaded in green.

Middle East, Afro-Asiatic

	-30210	-30203	-30196	-30182	-30160	-30071/70	-29949	-14011	-14010	-14009	-13915	-13913	-13910	-13907	-13806	-13779	-13744	-13730	-13603	-13495	-958	-943	-942	-931	-875	-678	666	
-30203	1																											
-30196	1	1																										
-30182	0.98	1	1																									
-30160	1	1	1	1																								
-30071/70	1	1	1	1	1																							
-29949	1	1	1	1	1	1																						
-14011	1	1	1	1	1	1	1																					
-14010	1	1	1	1	1	1	1	1																				
-14009	1	1	1	1	1	1	1	1	1	1																		
-13915	1	1	1	1	1	1	1	1	1	1	1																	
-13913	1	1	1	1	1	1	1	1	1	1	1	1																
-13910	1	1	1	1	1	1	1	1	1	1	1	1	1															
-13907	1	1	1	1	1	1	1	1	1	1	1	1	1	1														
-13806	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1													
-13779	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1												
-13744	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1											
-13730	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1										
-13603	0.70	1	1	0.77	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1									
-13495	0.95	1	1	0.77	1	1	0.78	1	1	1	1	1	1	1	1	1	1	1	1	1								
-958	0.96	1	1	0.97	1	1	1	1	1	1	0.97	1	1	1	1	1	1	1	1	1	1							
-943	1	1	1	0.88	1	1	0.12	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1						
-942	0.86	1	1	0.88	1	1	0.12	1	1	1	1	1	1	1	1	1	1	1	1	1	1	0.85	1					
-931	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1				
-875	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1			
-678	1	1	1	1	1	1	1	1	1	1	0.97	1	1	1	1	1	1	0.69	1	1	0.96	1	1	1	1	1	1	
666	0.97	1	1	0.97	1	1	0.57	1	1	1	1	1	1	1	1	1	1	1	1	1	0.98	0.99	1	1	1	1	1	
5579	0.81	1	1	0.64	1	1	1	1	1	1	0.97	1	1	1	1	1	1	1	1	1	0.92	0.86	0.10	0.08	1	1	0.77	0.75

Africa, Niger-Congo

	-30210	-30182	-30071/70	-29949	-14010	-13910	-13800	-13730	-13603	-13495	-943/42	-678	666
-30182	1												
-30071/70	1	1											
-29949	1	1	1										
-14010	1	1	1	1									
-13910	1	1	1	1	1								
-13800	1	1	1	1	1	1							
-13730	1	1	1	1	1	1	1						
-13603	1	1	1	1	1	1	1	1					
-13495	1	1	1	1	1	1	1	1	1				
-943/42	0.94	1	1	0.43	1	1	1	1	1	0.79			
-678	1	1	1	1	1	1	1	1	1	1	1		
666	1	0.96	1	0.14	1	1	1	1	1	1	0.97	0.96	
5579	1	0.91	1	0.73	1	1	0.69	1	0.92	1	0.16	0.92	0.18

Africa, Nilo-Saharan

	-30210	-30196	-30182	-30160	-29949	-13800	-13730	-13495	-958	-943/42	-678	666
-30196	1											
-30182	1	1										
-30160	1	1	1									
-29949	1	1	1	1								
-13800	1	1	1	1	1							
-13730	1	1	1	1	1	1						
-13495	0.07	1	1	1	1	1	1					
-958	1	1	1	1	1	1	1	1				
-943/42	1	1	1	1	1	1	1	1	1			
-678	1	1	1	1	1	1	1	1	1	1		
666	0.53	1	1	1	1	1	1	0.30	1	1	1	
5579	0.22	1	1	1	1	1	1	0.65	1	0	1	0.42

Middle East, Indo-European

	-30210	-30182	-30160	-29949	-13910	-13495	-958	-875	-678	666
-30182	1									
-30160	1	1								
-29949	1	1	1							
-13910	1	1	1	1						
-13495	1	0.34	1	1	1					
-958	1	1	1	1	1	1				
-875	1	1	1	1	1	1	1			
-678	1	1	1	1	1	1	1	1		
666	1	1	1	1	1	1	1	1	1	
5579	0.91	0.21	1	1	1	1	0.91	1	0.60	0.91

Europe/Asia, Indo-European

	-30210	-30182	-30160	-29949	-14028	-14011	-13910	-13495	-958	-943	-942	-875	-678	666
-30182	1													
-30160	1	1												
-29949	1	1	1											
-14028	1	1	1	1										
-14011	1	1	1	1	1									
-13910	1	1	1	1	1	1								
-13495	1	0.88	0.48	1	1	1	1							
-958	0.98	1	1	1	1	1	1	1						
-943	1	1	1	1	1	1	1	1	0.60					
-942	0.65	1	1	1	1	1	1	1	0.29	1				
-875	1	1	1	1	1	1	1	1	1	1	1			
-678	0.87	0.93	1	1	1	1	1	1	1	1	1	1		
666	0.97	1	1	1	1	1	1	1	1	1	1	1	1	
5579	0.97	0.58	1	0.79	1	1	1	1	0.97	1	1	1	0.70	0.97

Middle East/Asia, Altaic

	-30210	-30182	-30160	-29949	-14011	-13910	-13495	-958	-943/42	-875	-678	666
-30182	1											
-30160	1	1										
-29949	1	1	1									
-14011	1	1	1	1								
-13910	1	1	1	1	1							
-13495	1	1	1	0.47	1	1						
-958	1	1	1	1	1	1	1					
-943/42	1	1	1	1	1	1	1	1				
-875	1	1	1	1	1	1	1	1	1			
-678	0.80	1	1	1	1	1	1	1	1	1		
666	0.87	1	1	1	1	1	1	1	1	1	1	
5579	0.76	0.49	1	1	1	1	1	0.83	1	1	0.52	0.87

Appendix G: Publications

(Jones et al. 2013)

(Gallego Romero et al. 2012)

(Jensen et al. 2011)

(Gerbault et al. 2011)

REPORT

Diversity of Lactase Persistence Alleles in Ethiopia: Signature of a Soft Selective Sweep

Bryony L. Jones,¹ Tamiru O. Raga,² Anke Liebert,¹ Pawel Zmarz,¹ Endashaw Bekele,² E. Thomas Danielsen,³ Anders Krüger Olsen,^{3,5} Neil Bradman,^{1,4} Jesper T. Troelsen,³ and Dallas M. Swallow^{1,*}

The persistent expression of lactase into adulthood in humans is a recent genetic adaptation that allows the consumption of milk from other mammals after weaning. In Europe, a single allele (-13910^*T , rs4988235) in an upstream region that acts as an enhancer to the expression of the lactase gene *LCT* is responsible for lactase persistence and appears to have been under strong directional selection in the last 5,000 years, evidenced by the widespread occurrence of this allele on an extended haplotype. In Africa and the Middle East, the situation is more complicated and at least three other alleles (-13907^*G , rs41525747; -13915^*G , rs41380347; -14010^*C , rs145946881) in the same *LCT* enhancer region can cause continued lactase expression. Here we examine the *LCT* enhancer sequence in a large lactose-tolerance-tested Ethiopian cohort of more than 350 individuals. We show that a further SNP, $-14009T>G$ (ss 820486563), is significantly associated with lactose-digester status, and in vitro functional tests confirm that the -14009^*G allele also increases expression of an *LCT* promoter construct. The derived alleles in the *LCT* enhancer region are spread through several ethnic groups, and we report a greater genetic diversity in lactose digesters than in nondigesters. By examining flanking markers to control for the effects of mutation and demography, we further describe, from empirical evidence, the signature of a soft selective sweep.

Lactase, the enzyme that digests the milk sugar lactose, persists into adult life in approximately 35% of the world's population.¹ This genetic trait of adult lactase persistence (LP [MIM 223100]) is a recent human adaptation permitting those who carry it to use animal milk more readily as a source of nutrition. This LP phenotype is in contrast to the ancestral mammalian phenotype shared by most of the human population where lactase is downregulated before adulthood.

LP is attributable to nucleotide changes in a regulatory region that acts as an enhancer of the expression of the gene encoding lactase, *LCT* (MIM 603202). This *LCT* enhancer is located in intron 13 of the neighboring gene, *MCM6* (MIM 601806), immediately upstream of *LCT* (for review see Ingram et al.¹). LP in Europe is generally attributable to a single allele (-13910^*T , rs4988235) that seems to have been under strong directional selection in the last 5,000–10,000 years.² The evidence for selection comes from the fact that the allele lies on an extended haplotype^{3,4} with low microsatellite diversity and is at a significantly higher frequency than expected for the age of the allele.^{1,4–6} Tests of haplotype homozygosity and population differentiation in genome-wide studies that focus on European samples show that the region of chromosome 2 containing *LCT* has one of the highest “signatures” of selection.⁷ In Africa and the Middle East, the situation is more complicated and three additional alleles (-13907^*G , rs41525747; -13915^*G , rs41380347;

-14010^*C , rs145946881) in the same *LCT* enhancer region have been reported to cause lactase persistence.^{8–16} In Tanzania and Kenya, one particular allele, -14010^*C , is at high frequency and gives a significant signal of positive selection in tests of haplotype homozygosity, with expansion of the -14010^*C allele dated to approximately 3,000–7,000 years ago.¹⁶ In some cases, however, including the Jaali from Sudan and the Somali camel herders from Ethiopia, several different LP alleles are associated with lactase persistence in the same ethnic group.¹⁵ Indeed, we observed that in these groups, the sequence of the enhancer region was much more diverse in lactose digesters than in nondigesters. Analysis of mitochondrial DNA, Y chromosome, and autosomal microsatellite variations demonstrated that this difference in diversity was not attributable to hidden population stratification.¹⁵ Although it is possible that not all of the described enhancer alleles are functional, we speculated that this difference in diversity had been influenced by what has been described as a soft selective sweep—the phenomenon by which several different variants of similar function rise in frequency simultaneously.^{17–19} Such soft selective sweeps might not be detectable by published methods of genome-wide detection of selection.^{20,21}

Here, we examine genetic diversity of the *LCT* enhancer in a larger lactose-tolerance-tested cohort (>350 individuals), which consists of volunteers from several ethnic groups in Ethiopia. We aim to determine whether any

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http://dx.doi.org/10.1016/j.ajhg.2013.07.008. ©2013 The Authors

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Digester Status	n (2 Allele Carriers Excluded)	-14011C>T	-14010G>C	-14009T>G	-13957A>G	-13938C>T	-13915T>G	-13910C>T	-13907G>C	-13806A>G	-13800G>T	-13730T>G	-13603C>T
rs/ss Number		rs4988233	rs145946881	ss820496563	ss820496514	ss831883152	rs41380347	rs41456145	rs4988235	rs41525747	ss820496565	rs144412793	rs56348046
H ₂ NP	14	0	0	2	1	0	3	0	4	0	0	1	0
Intermediate	44	0	0	3	0	1	5	2	2	2	0	2	0
Digester	278 (196)	1 (0)	3 (2)	22 (13)	1	0	74 (34)	2 (1)	1 (0)	44 (30)	6 (1)	0	8 (2)
Nondigester	376 (374)	1 (0)	0	6	1	0	6	7	0	2 (1)	10	1	17
p value		1	0.076	0.00008*	1	NA	9.63 × 10 ⁻²⁴ *	0.313	0.43	1.91 × 10 ⁻¹⁶ *	0.801	NA	0.310
p value (2 allele carriers excluded)		NA	(0.117)	0.00242*	NA	NA	9.50 × 10 ⁻¹² *	0.274	NA	5.06 × 10 ⁻¹⁴ *	0.108	NA	0.0264

The results reported here are from a total of 356 volunteers (712 chromosomes). To test association of the alleles independently, counts were also made after exclusion of all individuals who carry two different derived alleles (numbers in parentheses). Fisher's exact test two-sided p values are shown for the comparison of digesters and nondigesters. Asterisks (*) indicate statistically significant association after Bonferroni correction for 12 tests (threshold $p = 0.004$). Note that -14011T>G is present in one digester, but the second carrier, despite being a nondigester, also carried -13907G, so was presumably suffering from secondary loss of lactase. The location of -14011T immediately next to -14010G>C suggests the possibility of function.

further genetic variants are likely to be functional, whether the different Ethiopian groups in our cohort carry private mutations, and whether the pattern of increased diversity of the enhancer region in lactose digesters that we detected previously is widespread in Ethiopia. We examine this diversity in relation to that of flanking markers to control for the effects of mutation and demography to describe this signal of selection.

The Ethiopian participants were university students who undertook lactose-tolerance testing by a breath hydrogen method. Ethical approval was obtained from the University of Addis Ababa and UCLH (ref 01/0236). Each volunteer provided fully informed consent for the project and completed a questionnaire relating to their self-declared ethnicity, ancestry, language (also language and ethnicity of parents and grandparents), and questions about health and milk-drinking habits. Buccal samples were collected from all participants, followed by DNA extraction²² and sequencing. Data were obtained from 370 individuals. Volunteers were classified as lactose digesters, nondigesters, intermediate/indeterminate digesters, or hydrogen non-producers via a breath-hydrogen lactose-tolerance test (LTT) as described by Ingram and colleagues.^{14,15} Data from 14 individuals were excluded from the association study because of recent antibiotic use (which could potentially affect lactose-tolerance test results) or known family relationship between participants, resulting in a data set from 356 individuals.

A total of 139 (39%) of the volunteers from this Ethiopian cohort were classified as lactose digesters and 188 (53%) as nondigesters. A total of 22 (6%) gave intermediate measurements, and 7 (2%) produced no breath hydrogen throughout the test.

The volunteers were classified according to self-declared cultural identity (ethnicity). Volunteers with both parents and known grandparents from the same ethnic group were classified as Amhara, Oromo, Tigre, or Wolayita, and those with other or mixed ancestry were classified as "other" but clustered as far as possible by language family.

We sequenced a 500 bp DNA fragment across the *LCT* enhancer (for primers, cycling, and sequencing conditions, see Table S1 available online) and recorded a total of 13 variable sites. None of the loci showed significant deviation from Hardy-Weinberg equilibrium (data not shown). None of the frequent derived alleles were private to any particular ethnic group (see Table S2).

Counts of the derived enhancer alleles for all lactose digesters are shown in Table 1. The strong association of -13907G and -13915G with digester status is clearly confirmed in this cohort. Of the other previously characterized variants, -14010C was present in digesters only but at too low a frequency to give statistical significance in this cohort and -13010T was present in one digester who also carried a second enhancer allele. In this study, we show a statistically significant association of the -14009G variant with lactose-digester status ($p = 0.0024$, with a Bonferroni threshold of $p < 0.004$ for 12

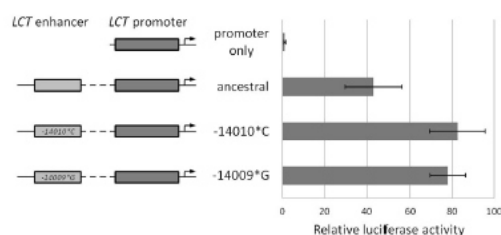


Figure 1. Transfection Experiment Showing the Effect of the -14009*G Variant on the Enhancer Activity in Caco-2 Cells
Promoter/enhancer constructs containing either the ancestral sequence, the -14009*G variant, or the -14010*C variant LCT enhancer sequence were transfected into Caco-2 cells together with a CMV-driven β -galactosidase expression plasmid. The cells were harvested and analyzed for luciferase and β -galactosidase activities 9 days after transfection. The luciferase expressions were corrected for transfection efficiency via the β -galactosidase activities. The -14010*C construct was used as a positive control. The bar chart shows luciferase expression relative to the "promoter only" construct (lacking the enhancer) and the error bars represent SDs ($n = 8$). Both the -14009*G and -14010*C enhancer sequences lead to an increased luciferase expression, which is significantly different from the ancestral sequence (-14009*G/-14010*G; $p = 0.000032$ and 0.000038 , respectively).

tests). Alleles -13730*G, -13806*G, and -13913*G were more frequent in nondigesters than in digesters, which indicates that these alleles do not cause LP. In this context, -13913*G is of particular interest because it is located very close to other known functional variants. All other alleles were too rare to assess.

To determine whether -14009*G has a functional effect on the LCT enhancer activity, the -14009*G mutation was introduced by site-directed mutagenesis into the 450 bp LCT enhancer region (-14,133 bp to -13,684 bp) and inserted into the pGL3hLPH1085 promoter construct with luciferase as a reporter, as previously described.^{8,11} Transfections were conducted with Caco-2 cells and the results are shown in Figure 1. A significant increase in luciferase activity was observed for the -14009*G construct in comparison with the ancestral sequence. This increase is comparable with the increase observed for the -14010*C variant (used here as a positive control), strongly supporting function, and our studies in progress confirm alteration of transcription factor binding (A.L., J.T.T., D.M.S., and colleagues, unpublished data). The fact that as many as six individuals who carry the allele were diagnosed as nondigesters does hint, however, that this allele may behave less efficiently than the others in vivo.

Several individuals carried one of the known LP variants and yet were classified as lactose nondigesters, whereas other individuals ($n = 17$) were classified as digesters, yet had no variant in the LCT enhancer. At least part of this disparity is probably attributable to one of the known limitations of this phenotypic test—the lack of control for loss of enzymatic function in the gut resulting from secondary damage.²³ However, the lactose digesters with no

enhancer variation and confirmed hydrogen production are more difficult to explain and might be real, suggesting that a different causal mechanism could be at work. A mutation in a different *cis*-acting region could be responsible for LP in these individuals, or perhaps even a *trans*-acting factor (although *trans*-acting variants are less likely to be causal because such mutations are more likely than *cis*-acting variants to have a pleiotropic effect²⁴). Epigenetic influences could also play a role in the persistence of lactase in some of these individuals.

In an initial analysis of the diversity of the LCT enhancer region, we found greater sequence diversity in the digesters than in the nondigesters (Nei's H for digesters 0.73 versus 0.023 for nondigesters; π 0.002 for digesters versus 0.0005 for nondigesters), which is consistent with our previous findings in Ethiopian Somali camel herders.¹⁵ To further explore the causes of this greater diversity in digesters, we sought to control for the possible effects of demography and mutation rate. To do this, we sequenced two control regions either side of the LCT enhancer that were unlikely in most cases to have been separated from the enhancer by recombination since the onset of the spread of these alleles (for primers, cycling, and sequencing conditions see Table S1).

We selected a 500 bp fragment 16 kb upstream of the LCT enhancer in intron 4 of *MCM6* and a 361 bp fragment 13 kb downstream of the enhancer, approximately 1 kb upstream of LCT exon 1. Assuming a recombination rate of 0.5 cM/Mb observed in families for that region (UCSC Genome Browser), 5% or fewer of the chromosomes are likely to have recombined during the last 300 generations (5,000 years). Intron 4 of *MCM6* is likely to have a similar chromatin state to that of intron 13 and, therefore, a similar vulnerability to germline mutation, thus doubling as a control region with similar mutation rate.

The subsequent analyses were conducted on samples with complete sequence data for all three regions, irrespective of phenotype classification. From the previously collected cohort of Ethiopian Somali camel herders,¹⁵ 81 individuals were included for comparison. The frequencies of sequence variants in the two control regions for digesters and nondigesters are shown in Table S3.

Haplotypes were inferred with the computer program PHASE,²⁵ and the results were then checked by visual inspection of the data. The haplotypes that were present in this cohort, excluding those haplotypes with fewer than three occurrences, are shown in Figure 2 in relation to our previous designations.²⁷ Figure S1 details the full set of haplotypes and indicates nonrecombinant and recombinant chromosomes. Only 7% of chromosomes show evidence of historic recombination between the flanking regions and the frequency of detectable recombinants is lower for the chromosomes carrying the derived enhancer alleles (3.9%). As previously reported, -13915*G is found on a C, or a closely related haplotype (of which 85 out of 88 are identical across all three regions), and -13907*G is on an extended A, or closely related

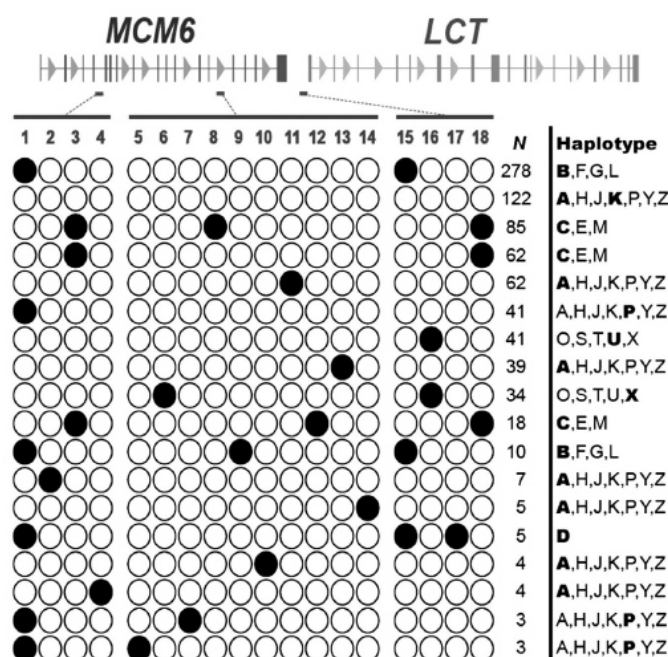


Figure 2. Phased Haplotypes for the Lactase Enhancer and Two Flanking Regions, in Intron 4 of *MCM6* and 1 kb Upstream of *LCT* in a Cohort of 422 Ethiopian Individuals with No Known Shared Ancestry to the Grandparental Level, for whom Full Sequencing Data Were Available

Haplotypes that occurred <3 times ($n = 21$) are omitted. The variable sites are numbered and refer to the following chromosome positions relative *LCT*: 1, -30210*G; 2, -30203ins; 3, -30182*G; 4, -29949*G; 5, -14010*G; 6, -14009*G; 7, -13957*G; 8, -13915*G; 9, -13913*G; 10, -13910*G; 11, -13907*G; 12, -13806*G; 13, -13730*G; 14, -13603*G; 15, -958*G; 16, -942del; 17, -875*G; 18, -678*G. n indicates the number of chromosomes for which that haplotype was inferred. The lettered haplotypes that are shown refer to the haplotypes previously reported by Hollox et al.²⁷ Those that are shown in bold are the most likely haplotype according to previously reported distributions and examination of additional alleles (unpublished data).

Figure 3 shows the distribution of nucleotide diversity values for each region. For the enhancer region, in all cases, π was greater in the digesters

haplotype, in 62 out of 63 cases (Figures 2 and S1). The -14009*G allele occurred in 34 out of 36 cases on a haplotype that has a deletion at -942, which, according to our previous haplotype designations, could be an O, S, T, U, or X type haplotype. Typing position 5579 of the *LCT* cDNA sequence (rs2278544) of a number of the chromosomes with the -14009*G showed that they carry a C nucleotide, suggesting that this is most probably an X, rather than the more common U, haplotype.²⁷

As an additional check that the enhancer region in intron 13 is not intrinsically more mutable than the other regions that we have examined, we compared the divergence of the three regions from primate sequences by using the sequences available from the UCSC Human Genome Browser. We observed that the divergence of the intron 13 region is in fact less than that of the other two regions. For example, in comparison with the rhesus macaque, the values obtained were 5.9%, 5.2%, and 6.3% for control region 1, the enhancer sequence, and control region 2, respectively.

We then compared haplotype diversity (H) and nucleotide diversity (π) of all three sequence regions (Table 2) in the digesters and nondigesters from the Amhara, Tigre, Oromo, and Wolayita and the previously collected Somali. Individuals of mixed ancestry or those belonging to groups with fewer than 20 individuals in our data set, for the purposes of this analysis were grouped together, irrespective of language family, as "all other Ethiopians."

than the nondigesters. We assessed the significance of the difference in π for each ethnic group by permutation analysis in which the haplotypes were drawn and assigned to digesters and nondigesters in 50,000 random permutations. A conservative two-tailed p value was computed as the proportion of permutations with an absolute π difference equal to or larger than the observed π difference between digesters and nondigesters. For the enhancer, there was a significant difference in π between digesters and nondigesters for all ethnic groups ($p < 0.005$) (Table S4). Similar comparison of the control sequences showed just one ethnic group with a significant π difference (the Oromo for the control region in intron 4, $p = 0.0002$).

Haplotype diversity of the enhancer region was also significantly greater in digesters than nondigesters for all ethnic groups (as determined by the test H_{diff} ,²⁶ Table S4). This distribution is depicted in Figure S2. As for the π analysis, the only significant difference in the control sequences was the intron 4 region in Oromo ($p = 0.0028$). However, even if not statistically significant, it is noteworthy that in most cases the diversity of both the flanking regions is slightly lower in the nondigesters than digesters of the same group. This effect, which is particularly prominent in the Somali group, is probably a consequence of the very high prevalence throughout Ethiopia of a long haplotype that does not carry any derived LP-associated alleles (the first on Figure 1, and see the blue haplotype in Figure S3).

Table 2. Comparative Diversity Data for the Three Regions Sequenced

Region	Ethnic Group	Digester Status	n	Control Region 1-Intron 4 MCM6				Enhancer - Intron 13 MCM6				Control Region 2-Upstream of LCT			
				Seg Sites	N Haplo	H	π	Seg Sites	N Haplo	H	π	Seg Sites	N Haplo	H	π
Amhara		nondigester	62	3	4	0.672	0.0019	4	5	0.185	0.00038	3	5	0.694	0.0026
		digester	32	2	3	0.669	0.0017	5	6	0.595	0.00141	3	4	0.736	0.0028
Oromo		nondigester	78	3	4	0.556	0.0013	5	6	0.261	0.00055	4	6	0.671	0.0027
		digester	54	3	5	0.706	0.0021	7	8	0.705	0.00185	4	6	0.716	0.0029
Tigre		nondigester	30	3	4	0.522	0.0014	1	2	0.067	0.00013	3	4	0.646	0.0023
		digester	48	2	3	0.635	0.0015	4	5	0.704	0.00182	3	4	0.668	0.0023
Wolayita		nondigester	24	2	3	0.565	0.0013	1	2	0.083	0.00017	3	4	0.663	0.0022
		digester	16	2	3	0.667	0.0016	4	5	0.717	0.00180	3	4	0.742	0.0027
Somali		nondigester	98	3	4	0.559	0.0013	3	4	0.223	0.00046	3	4	0.588	0.0021
		digester	32	3	4	0.688	0.0017	5	6	0.714	0.00182	3	4	0.718	0.0026
Other		nondigester	162	4	5	0.621	0.0015	9	10	0.312	0.00067	4	5	0.686	0.0025
		digester	132	2	4	0.652	0.0016	10	11	0.748	0.00203	4	5	0.732	0.0029

Using the fully phased data set, measures of genetic diversity were calculated with DnaSP software, using those samples with a clear diagnosis of either lactase-digester or -nondigester status. Note that for the enhancer region, the numbers of segregating sites, numbers of haplotypes, and calculated values for H (haplotype heterozygosity) and π (nucleotide diversity) are in each case larger in the digesters than nondigesters. These analyses were conducted on samples for which uniform ancestry was recorded and a single additional group was composed of all others. Two simple indel polymorphisms (one in intron 4 and one in the upstream LCT region) are considered as SNPs in the calculations. N = number of chromosomes. Detailed statistics are shown in Table S4.

Thus we confirm our previous observation on the diversity of the LCT enhancer sequence region and show with a cohort of independent samples that there is significantly greater nucleotide and haplotype diversity in the enhancer region in digesters than in nondigesters.

By sequencing two flanking regions, which have followed the same demographic history as the enhancer over the last 300 generations, both control sequences have rather high diversity in nondigesters as well as in digesters. Indeed, it is the lack of diversity in the enhancer sequence of nondigesters that is noteworthy and this seems to reflect the general conservation of this sequence across primates. Between positions -14028 and -13800,

the sequences are approximately 93.5% identical across humans, chimpanzee, gorilla, orangutan, gibbon, baboon, and rhesus macaque, and percentage identity declines on either side of this (see Figure S4). Thus, this sequence region may have been under evolutionary constraint because of its function as a regulatory element for lactase expression in infant mammals. This constraint appears to have been overcome in some human populations, presumably because of the benefit of allowing adult lactase expression.

This study brings the total of confirmed LP causal variants to five (-14010*C, -14009*G, -13915*G, -13910*T, and -13907*G). Their close location, all within the most conserved part of the 450 bp segment known to have

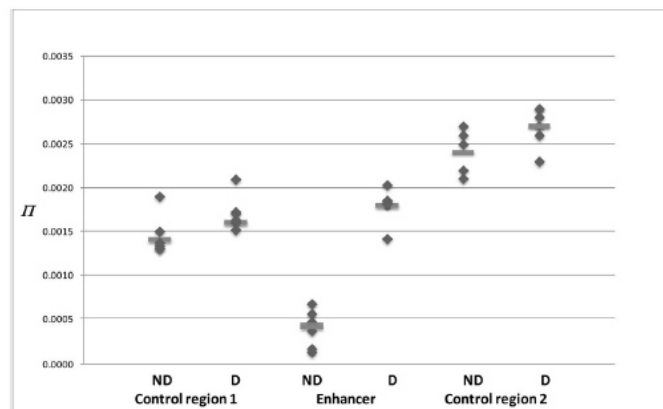


Figure 3. Comparison, in Nondigesters and Digesters, of Nucleotide Diversity π Measured across the Three Sequence Regions

Data points show the five ethnic groups tested: Amhara, Tigre, Oromo, Wolayita, Somali, and "other Ethiopians" as a single group. Red horizontal bars show median values. See Table 2 for n values. Abbreviations are as follows: D, digester; ND, nondigester.

enhancer function, suggests that the functional region is smaller than the sequence that we have tested and this will be explored further in future studies.

The coexistence of all five alleles in Ethiopia goes some way to explaining the greater nucleotide diversity within the *LCT* enhancer in intron 13 of *MCM6* in digesters. Ethiopia has been a crossroads of human migrations in the last 5,000 years since the LP alleles are likely to have come under selection, and studies on other African and Middle eastern populations (B.L.J., D.M.S., and colleagues, unpublished data) show quite different geographic distributions, with overlap in Ethiopia, suggesting that their origins are all different, but determining where these were and how they spread is likely to be difficult. The combination of mutation, large effective population size, migration, and selection has been shown to be important in generating this kind of pattern of diversity, namely parallel selection of multiple alleles of similar function, a so-called soft selective sweep.¹⁷ Here we confirm this unusual pattern of diversity in the *LCT* enhancer region in Ethiopia, and by testing flanking sequences we control, at a chromosomal level, for possible differences in migrational history, effective population size, and mutation rate between the digesters and nondigesters. Because increased genetic diversity in digesters is localized to the *LCT* enhancer, we can infer that recent selection is acting on this small relatively conserved functional sequence region. Selection has the effect of increasing the frequency of the background haplotypes on which the derived alleles occur, though the impact on diversity of the flanking sequences is not large. This pattern contrasts with that observed from the hard selective sweep seen for the "European" *LCT* enhancer allele, where a single extended haplotype is present in at least one copy in digesters, whereas haplotype heterozygosity is significantly higher for the nondigesters^{3,4,28} (D.M.S. and colleagues, unpublished data). This study, therefore, provides important pointers toward describing the sequence features of such motifs in other areas of the genome.

Supplemental Data

Supplemental Data include four figures and four tables and can be found with this article online at <http://www.cell.com/AJHG/>.

Acknowledgments

This work was funded by the MRC UK (MRC DTA studentship for B.L.J.), the European Union (Marie Curie ITN FP7 Framework Programme grant, LeCHE, Grant ref 215362-2 to A.L.), the Annals of Human Genetics (B.L.J. and A.L.), and Melford Charitable Trust (studentship for T.O.R.). N.B. is the settlor and senior trustee of Melford Charitable Trust. Neither N.B. nor the charitable trust has any intellectual property or other rights with respect to the results of the study. We thank Mari Wyn Burley and Ranji Araseretnam for technical help, Mima Kovacevic and Ripudaman Bains for their help with data handling, and Ed Hollox for helpful discussion.

Received: March 28, 2013

Revised: June 7, 2013

Accepted: July 2, 2013

Published: August 29, 2013

Web Resources

The URLs for data presented herein are as follows:

DnaSP, <http://www.ub.edu/dnasp/>

Online Mendelian Inheritance in Man (OMIM), <http://www.omim.org/>

UCSC Genome Browser, <http://genome.ucsc.edu>

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Herders of Indian and European Cattle Share Their Predominant Allele for Lactase Persistence

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Associate editor: Sarah Tishkoff

Abstract

Milk consumption and lactose digestion after weaning are exclusively human traits made possible by the continued production of the enzyme lactase in adulthood. Multiple independent mutations in a 100-bp region—part of an enhancer—approximately 14-kb upstream of the *LCT* gene are associated with this trait in Europeans and pastoralists from Saudi Arabia and Africa. However, a single mutation of purported western Eurasian origin accounts for much of observed lactase persistence outside Africa. Given the high levels of present-day milk consumption in India, together with archaeological and genetic evidence for the independent domestication of cattle in the Indus valley roughly 7,000 years ago, we sought to determine whether lactase persistence has evolved independently in the subcontinent. Here, we present the results of the first comprehensive survey of the *LCT* enhancer region in south Asia. Having genotyped 2,284 DNA samples from across the Indian subcontinent, we find that the previously described west Eurasian -13910 C>T mutation accounts for nearly all the genetic variation we observed in the 400- to 700-bp *LCT* regulatory region that we sequenced. Geography is a significant predictor of -13910*T allele frequency, and consistent with other genomic loci, its distribution in India follows a general northwest to southeast declining pattern, although frequencies among certain neighboring populations vary substantially. We confirm that the mutation is identical by descent to the European allele and is associated with the same >1 Mb extended haplotype in both populations.

Key words: positive selection, India, haplotype, lactase persistence, pastoralism.

Introduction

Lactase persistence (MIM #223100) is an exclusively human trait (Johnson et al. 1974), inherited in a dominant Mendelian fashion (Ferguson and Maxwell 1967; Sahi et al. 1973), and defined as the ability to maintain post-weaning production of the enzyme lactase—phlorizin hydrolase (LPH), which hydrolyzes the milk sugar lactose into its component monosaccharides. In all other mam-

mals tested, LPH production diminishes significantly after weaning and is never resumed (Plimmer 1906; Sebastio et al. 1989; Buller et al. 1990; Lacey et al. 1994). Initially considered to have a worldwide distribution, data collected from the mid 1960s and onward revealed that lactase persistence is geographically restricted and not the ancestral condition in humans. This trait is most common in northwestern Europe (Cuatrecasas et al. 1965; Bayless and

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Mol. Biol. Evol. 29(1):249–260. 2012 doi:10.1093/molbev/msr190 Advance Access publication August 11, 2011

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Rosensweig 1966; Simoons 1978; Davey Smith et al. 2009; Ingram, Mulcare, et al. 2009) and decreases in frequency east and south of there, reaching near-zero frequencies east of the Indian subcontinent (Ingram, Mulcare, et al. 2009; Itan et al. 2010). It is also present at moderately high frequencies in certain ethnic groups from sub-Saharan Africa, the Near East and the Mongolian plateau that have a history of milk consumption and pastoralism (Simoons 1970a; McCracken 1971; Durham 1991).

West Eurasian lactase persistence has been attributed to a single nucleotide change located approximately 14-kb upstream of the *LCT* gene (rs4988235, henceforth -13910 C>T; Enattah et al. 2002; Troelsen et al. 2003). In populations examined so far, the -13910*T allele is associated with a single extended haplotype that spans about 1 Mb (Poulter et al. 2003; Bersaglieri et al. 2004). The only exceptions to this are some rare chromosomes present in a group of populations living in the Eastern European Plain, where an argument has been made for the convergent origin of the -13910*T substitution (Enattah et al. 2007), although in this case, the data are also compatible with recombination or errors in phase inference. In spite of this strong genotype–phenotype association in Europe, -13910 C>T does not explain the observed patterns of lactose tolerance in Africa (Mulcare et al. 2004) and some other regions of the Old World (Itan et al. 2010). Recent studies have instead identified other variants of probable functional significance in the same small (approximately 100 bp) genomic region with known enhancer function (Olds and Sibley 2003; Lewinsky et al. 2005) among pastoralist, milk-drinking groups in sub-Saharan Africa (Ingram et al. 2007; Tishkoff et al. 2007; Ingram, Raga, et al. 2009) and Saudi Arabia (Enattah et al. 2008). Compared with the wide geographic distribution of -13910 C>T, the African and Arabian variants exhibit somewhat narrower and overlapping geographic distributions (Itan et al. 2010).

These multiple instances of phenotypic convergence imply the existence of a strong selective advantage to being lactase persistent in cultures where milk is commonly available. Indeed, genomic scans for signatures of natural selection have identified the 1-Mb region on chromosome 2 surrounding -13910*T as harboring signals of recent strong positive selection centered around -13910*T itself (Bersaglieri et al. 2004; Voight et al. 2006; Sabeti et al. 2007); the same is true of the >2 Mb surrounding -14010*C, one of the African variants (Tishkoff et al. 2007). In addition, variation in linked microsatellites (Coelho et al. 2005), an absence of the -13910*T allele in a sample of early Neolithic central European skeletons (Burger et al. 2007) and demic (Gerbault et al. 2009), as well as spatially explicit computer simulations (Itan et al. 2009) have all added support to the case for a strong selective advantage. Other studies have documented the occurrence of several different mutations in lactose-digesting members of a single ethnic group, suggestive of a recent soft selective sweep (Ingram, Raga, et al. 2009).

The likely selective advantage has been argued to be conditional on postweaning access to abundant sources of milk (Simoons 1970a; McCracken 1971; Durham

1991), something that would not have been widely available to humans prior to the domestication of cattle, sheep, or goats and the development of dairying practices. It has therefore been argued that the generalized adoption in Europe of a dairying culture during the Middle Neolithic, after the domestication of taurine cattle in the Near East, led to positive selection for lactase persistence and thus to the trait's present-day high frequency in populations from the region (Clutton-Brock 1999; Troy et al. 2001; Evershed et al. 2008; Itan et al. 2009). Its presence in sub-Saharan Africa may at least in part be explained by the adoption of pastoralism and dairying by north African groups that subsequently expanded south as indicated by the archaeological record and the pattern of expansion of their cattle (Hanotte et al. 2002), but the occurrence of several novel alleles in East Africa and the Arabian peninsula indicates convergent evolution. One with wider distribution, -13915*C, is most frequent in Saudi Arabian camel herders, who also exhibit a high frequency of lactase persistence (Enattah et al. 2008), suggesting that it may have originated in the Arabian peninsula, although the other known mutations (13907*G and 14010*C) are likely to have originated in Africa (Ingram et al. 2007; Tishkoff et al. 2007). The Indian subcontinent, at the eastern extreme of the phenotypic distribution (Simoons 1970b), remains a glaring lacuna in current knowledge. India is the world's largest milk producer today (Food and Agriculture Organization of the United Nations 2009), and zebu cattle, *Bos indicus*, a species distinct from west Eurasian *Bos taurus*, was probably domesticated in the Indus Valley around 7,000 years before present (YBP) (Meadow 1993; Loftus et al. 1994; Baig et al. 2005; Chen et al. 2010). A second milking animal, the water buffalo (*Bubalus bubalis*), accounts for over 50% of the milk produced in India (Kumar et al. 2007) and has similar levels of milk lactose to cattle (Jensen 1995), although specific details of its domestication time and archaeological context in India are lacking. Yet, despite being host to over 1 billion people and having a well-documented history of consumption of dairy products (Simoons 1970b), little is known about the distribution of lactase persistence beyond a flurry of studies conducted in the 1970s (Desai et al. 1970; Swaminathan et al. 1970; Gupta et al. 1971; Reddy and Pershad 1972; Tandon et al. 1981). These data suggest that phenotypic lactase persistence could be as high as 0.73 in some north Indian populations and rare or absent from some groups in the south of the country, predicting an overall north–south phenotypic frequency gradient, as well as a moderately high countrywide frequency of the phenotype. With the exception of a single recent study in which variation at the -13910 position was typed in two mixed urban populations (Babu et al. 2010), there exists no genotypic data related to the lactase persistence phenotype in India.

We have undertaken the first countrywide screening of DNA samples from all major language groups and geographic regions of India ($n = 2284$) and sequenced the region around -13910 to identify polymorphisms that are known to be or might be associated with lactase persistence.

In order to distinguish between shared and independent origins of mutations and to determine the extent of potentially positive selection, we genotyped a subset of 199 samples for over 1,000 single nucleotide polymorphisms (SNPs) surrounding the *LCT* locus on chromosome 2. These data are used to address four aspects of the genotypic basis for milk drinking among south Asians—first, what is the distribution of the west Eurasian -13910*T allele as well as that of any other mutations in the 700-bp region surrounding -13910 C>T that may be segregating in the country; second, how does this distribution fit previously reported frequencies of the lactase persistence phenotype; third, is the trait associated with linguistic, geographical, or ancestral subsistence factors; and lastly, is there genetic evidence for positive selection acting on lactase persistence alleles in the Indian subcontinent.

Materials and Methods

Sample Composition

Our sample consisted of 2,264 individuals from 105 distinct tribal and caste populations representing all five major language families and 22 of 28 Indian states, and a single Union territory, as well as a sample of 20 Tharu individuals from Nepal, for a total sample size of 2,284 unrelated individuals. States were grouped by previously described geographic regions (Sahoo et al. 2006); the Nepali samples were grouped with north India. A more detailed description of the samples is provided in supplementary table 1 (Supplementary Material online). All samples were collected with the informed written consent of the donors, and the study was approved by the Institutional Ethical Committee of the Centre for Cellular and Molecular Biology in Hyderabad.

Data Sequencing and Genotyping

Polymerase chain reaction (PCR) amplification was carried out using previously described conditions and primer pairs MCM6i13-LAC14CL2 and MCM6i13-MCM778, which respectively yield 427- and 706-bp amplicons (Ingram et al. 2007). We sequenced the amplified region surrounding the -13910 C>T locus in all 2,284 individuals using an ABI Prism 3730XL DNA Analyzer and BigDye terminator chemistry (Applied Biosystems, Foster City, CA). Sequence data were analyzed using Sequencher 4.8 (Genecodes, Ann Harbor, MI); all sequences were inspected visually and scored at all identified variable sites. All newly determined polymorphic positions and singletons were confirmed by reamplification and sequencing from the opposite strand. Similarly, the allelic status of individuals with the -13910*T allele was confirmed by an independent PCR. Population-, geographic-, linguistic-, and continental-level exact tests of Hardy–Weinberg equilibrium were carried out using a previously described method (Wigginton et al. 2005). Wilcoxon rank sum and Kruskal–Wallis tests were used to search for significant effects in population allele frequencies of subsistence, linguistic, and geographic factors. Chi-square tests were used to confirm associations between the presence/absence of lactase persistence alleles and geo-

graphic and linguistic factors and Mantel tests to test for interactions between geographic origin and linguistic affiliation known to covary across India. The linguistic distance matrix ranked populations that spoke languages from the same language family as having a distance of 0, whereas populations who spoke languages from different families had a distance of 1. We deemed the 0–1 approach most suited to ensuring the independence of the two matrices as the inclusion of more detailed linguistic information (i.e., subbranches) would force the introduction of additional arbitrary parameters when calculating distances between subbranches of different language families.

In all cases, we estimated a predicted phenotypic frequency of lactase persistence due to the given mutation assuming dominance using the Hardy–Weinberg formula $2pq + p^2$, where p is the frequency of the lactase persistence-associated allele and $q = 1 - p$, observing no deviations from expectations in any of the individual population groups. To reduce noise due to small sample size effects, however, we excluded from all population-level analyses 25 groups that had sample sizes <10. Data from a total of 81 populations were therefore included in downstream analyses. Then, we used the GenoPheno Monte Carlo method (Mulcare et al. 2004; Itan et al. 2010) to test whether our genotypic data could explain observed (from published literature) phenotype frequencies throughout the subcontinent, after factoring in sampling errors at both the genotypic and the phenotypic level, as well as type 1 and type 2 error rates in phenotype testing methods (usually breath hydrogen or blood glucose) as described by Mulcare et al. (2004) and Itan et al. (2010). All statistical analyses were performed using R 2.10 (R Core Development Team 2009). Published phenotype frequencies based on lactose tolerance testing of Indian populations without genotype information were retrieved from the online GLAD database (<http://www.ucl.ac.uk/mace-lab/resources/glad>, 2011 August 15). Surface interpolation and maps were generated by the PyNGL Python module (<http://www.pyngl.ucar.edu/>, 2011 August 15).

Haplotype Data Analysis

One hundred and ninety-nine Indian individuals in our sample (supplementary table 1, Supplementary Material online) were also genotyped using either the Illumina Infinium 650K ($n = 152$) or the 610K SNP ($n = 47$) platforms (Illumina, San Diego, CA). We retrieved unphased SNP data for the 5 Mb surrounding -13910 C>T (hg18 chr2:134,000,000–139,000,000) for these individuals as well as for the 506 unrelated individuals of European ($n = 156$), Near Eastern ($n = 160$), or Pakistani ($n = 190$) ancestry included in the Human Genome Diversity Panel—Centre d'Étude du Polymorphisme Humain (HGDP-CEPH), who have been previously genotyped on the 650K platform (Li et al. 2008). Data were phased using Beagle 3.1 (Browning and Browning 2007). Although -13910 C>T is not included in either the 650K or the 610K platforms, the HGDP-CEPH populations have been previously genotyped at this position (Bersaglieri et al. 2004), so we were able to include

it in our data set. The 5-Mb region spanned 1,152 SNP loci present in both SNP chips genotyped in 705 individuals.

By the use of a solid spine method (Barrett et al. 2005), we identified in the phased data a 60-kb linkage block spanning the lactase enhancer, containing 15 SNPs, which we used to define short-range haplotypes in the region surrounding the enhancer (supplementary table 2, Supplementary Material online). We then used the program NETWORK 4.5.1.6 to create an unrooted median joining network (Bandelt et al. 1999) from these SNPs (fig. 2). There is no overlap between markers included in the Illumina 650K and those originally employed to define the core LCT gene haplotypes (Hollox et al. 2001). To associate the 60-kb linkage block haplotypes with the established core haplotype nomenclature, we used the trio samples from HapMap CEU individuals, who have been genotyped for both sets of markers as had other members of their two- or three-generation families, which had allowed us to determine accurately the phased haplotypes from family structure.

We employed the program Sweep (Sabeti et al. 2007) to calculate EHH and relative EHH (REHH) scores for the 15-SNP-long core haplotypes in our four continental population groups after exclusion of 106 SNPs not annotated in the UCSC human genome build 16, which is the genomic build employed by Sweep. We further chose to calculate separate scores for Indo-European speakers and non-Indo-European speakers to test for differences between the two groups in light of a possible association between geography, linguistic affiliation, and the frequency of the lactase persistence allele uncovered by our analyses.

Results

-13910 C>T in India

Sequencing of the 427- and 706-bp PCR amplicons, approximately 14-kb upstream of the LCT gene in a total of 2,284 individuals, revealed that the derived -13910*T allele has the highest frequency among the observed mutations as well as the widest distribution throughout the Indian subcontinent (table 1, supplementary table 1, Supplementary Material online). Its frequency ranges from 0.8% among the Tibeto-Burman speakers to 18.4% among Indo-European speakers, with west India showing the highest frequency of the derived allele; it has an average countrywide frequency of 10.3%. Seven other segregating polymorphic sites were also observed, one of which (-13779 G>C) has an overall frequency of 2.4% in India (supplementary table 3, Supplementary Material online), whereas the remaining six mutations combined have an overall frequency of roughly 1%.

A broad geographic distribution map of -13910*T allele frequency in south Asia is presented in figure 1. Despite the general north to south and west to east frequency gradient, the occurrence of the -13910*T allele showed large differences between some neighboring groups (supplementary table 1, Supplementary Material online). Population-level mean allele frequency is 0.09 (standard deviation [SD] \pm 0.118); it is absent in almost a third of the population samples that met our criteria for minimum sample size of 10 and peaks in the Ror of Haryana (0.489, n = 46), a tradi-

tional milk-drinking people who herd water buffalo and a predicted frequency of the lactase persistence phenotype of 0.739. Of further curious note is the single Great Andamanese individual with a C/T genotype, suggestive of recent mainland Indian admixture (Thangaraj et al. 2003; Reich et al. 2009).

No single population under study showed significant departures from Hardy-Weinberg expectations (table 1). However, when pooled either by linguistic or by geographic criteria, disequilibrium is apparent in south and west India and in Dravidian and Indo-European speakers, indicating a Wahlund effect (Wahlund 1928) due to nonnegligible population structure. When considering all India as a single population, deviations from equilibrium due to the excess of homozygotes are pronounced (exact P = 2.83×10^{-13}). This deviation is more pronounced than in 156 unrelated European HGP-CEPH samples (exact P = 0.0007). This finding is consistent with genome-wide patterns of generally higher population differentiation in India than in Europe (Reich et al. 2009) as it stems primarily from an excess of homozygotes of both genotypes and corresponding shortage of heterozygous individuals.

We grouped populations by their linguistic or geographic affiliation (table 1, supplementary table 1, Supplementary Material online) and carried out Kruskal-Wallis tests to assess the effect of these factors on -13910*T allele frequencies. Both geography (P = 1.853×10^{-6} , n = 81) and language (P = 1.74×10^{-6} , n = 81) exhibit significantly nonrandom association with allelic frequencies, whereby groups from west India, or those that speak Indo-European languages are more likely to carry the -13910*T allele than any other group. It is difficult to quantify the degree of autocorrelation between our two main explanatory variables, but they are well known to covary (supplementary table 1, Supplementary Material online); a further Kruskal-Wallis test applied only on data from Indo-European speakers showed that geography remains significant (P < 0.01) even when controlling for language. Because there is no geographic region covered by our data with a sufficiently large number of populations of diverse linguistic affiliations, we cannot test the reverse hypothesis. Contingency table χ^2 tests further support the lack of independence between the presence of lactose tolerance in populations (i.e., freq T > 0) and linguistic affiliation (P = 6.15×10^{-5} , degrees of freedom [df] = 5) or geographic origin (P = 3.24×10^{-4} , df = 6); they also strongly support the lack of independence between our two main predictors (P = 3.85×10^{-29} , df = 30). Finally, Mantel tests suggest that geographic origin is having a significant effect on population frequencies of -13910*T (P = 0.005), although the effect of linguistic affiliation is negligible (P = 0.31); a partial Mantel test for the effects of linguistic affiliation holding geography constant further supports this conclusion (P = 0.707).

Other Enhancer Region Alleles Identified

Apart from -13910 C>T, we found seven other segregating sites in our samples with a combined frequency of the derived alleles 0.035. Four have been previously described; the

Table 1. Sample-Wide Allele Frequencies of Previously Published Lactase Persistence Associated Variants by Linguistic and Geographic Affiliation.

	<i>n</i>	Observed Allele Frequency					HWE <i>P</i> Value —13910*T
		—14010*C ^a	—13915*G rs41380347	—13910*T rs4988235	—13907*G rs41525747	Other Observed Variants ^b	
Linguistic affiliation ^c							
Andamanese	24	0	0	0.021	0	0	1
Austroasiatic	331	0	0	0.011	0	0	1
Dravidian	853	0	0	0.076	0	0.046	1.47 × 10 ^{−4}
Indo-Europeans	896	0	0	0.184	0	0.016	3.24 × 10 ^{−4}
Isolate	60	0	0	0.025	0	0.042	1
Tibeto-Burman	120	0	0	0.008	0	0	1
Total	2284	0	0	0.103	0	0.025	2.83 × 10 ^{−13}
Geographic region ^d							
West	468	0	0	0.213	0	0.019	2.71 × 10 ^{−4}
North	290	0	0	0.159	0	0.022	0.268
East	310	0	0	0.018	0	0.003	1
North East	139	0	0	0.007	0	0	1
Central	179	0	0	0.056	0	0.003	0.430
South	864	0	0	0.085	0	0.047	5.99 × 10 ^{−4}
Andaman and Nicobar islands	34	0	0	0.015	0	0	1
Total	2284	0	0	0.103	0	0.025	2.83 × 10 ^{−13}

NOTE.—^aThe rs id associated with this position, rs4988233, has alleles C and T, the derived T allele was not detected in our sample.

^b More details on the sites grouped under “other observed variants” are given in the text and in supplementary tables 3 and 4 (Supplementary Material online).

^c Linguistic affiliations of the 106 sampled populations are given in supplementary table 1 (Supplementary Material online).

^d Geographic regions are defined as in Sahoo et al. (2006).

remaining three are novel (table 1; supplementary tables 3 and 4, Supplementary Material online). Of these seven sites, the mutation -13779 G>C, previously reported in a single lactose intolerant Somali individual (Ingram, Raga, et al. 2009), is the most common, with a countrywide sample frequency of 0.024. Its distribution is primarily restricted to eastern Kerala, where it reaches its highest frequency in the hunter-gatherer group Kattunaikkan (0.146, $n = 41$) and in the nearby Nilgiri Hills of Tamil Nadu. Its frequency in the Toda, who are pastoralists and herders of water buffaloes, is 0.091 ($n = 55$), much lower than the observed value for -13910*T for the same group (0.445) but still worthy of note. Also of interest is the -13915

T>C allele, which we have previously found in Muslim populations from southern India (Easwarkhanth et al. 2010) and which we report here at a low frequency in other populations from the same region, again including the Toda. This is distinct from the previously described -13915 T>G mutation, which was not found in any of the samples.

Reconciling Genotype and Phenotype

On the basis of previously collected lactase persistence phenotype data (Desai et al. 1970; Swaminathan et al. 1970; Gupta et al. 1971; Reddy and Pershad 1972; Tandon et al. 1981), the average observed frequency of lactase persistence in India appears to be roughly 0.40 (supplementary fig. 1, Supplementary Material online). However, based on our -13910*T allele frequency data, the predicted countrywide phenotype frequency in our sample is only 0.196. The same is true when we calculate the mean predicted phenotypic frequency at the population group level, 0.158 (SD ± 0.191)—the median population value is likewise low, 0.089. In our data, point estimates of phenotype frequency explained by -13910*T allele frequency only reach or exceed 0.40 in 11 of the 81 sampled groups and are less than 0.1 in 45 of the 81 groups. Given that we observe an absence of -13910*T alleles in many of these samples, phenotype frequencies could in many cases be close to zero; 95% confidence intervals for all populations are given in supplementary table 1 (Supplementary Material online). Even when we assume that all observed mutations in the region under study are causative and dominant, our predicted countrywide phenotype frequency only increases to 0.24, again substantially lower than 0.40 (supplementary table 5, Supplementary Material online). These results suggest either that there are other major genetic variants

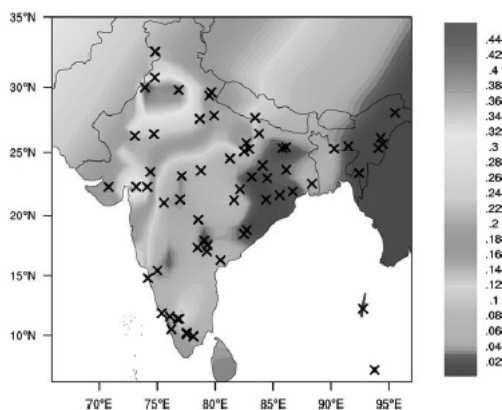


FIG. 1. Distribution of -13910 C>T in India. Crosses represent sample locations. Colors and color key show the frequency of the -13910*T allele estimated by surface interpolation.

Table 2. The Effect of Pastoralism on HWE.

	Pastoralists Included ^a	Pastoralists Excluded
Dravidians	1.47×10^{-04}	0.134
Indo-Europeans	3.24×10^{-04}	0.084
South India	5.99×10^{-04}	0.154
West India	2.71×10^{-04}	0.153
All India	2.83×10^{-13}	3.68×10^{-5}

NOTE.—The table shows *P* values for HWE for all populations grouped by geographic region (a) with pastoralists included and (b) with pastoralists excluded.

^a Only those groups that originally included pastoralists are shown in this comparison. All values are from exact HWE tests.

outside our sequencing range that are causative of the lactase persistence phenotype or that previous studies have overestimated the actual phenotype frequency in India. However, our GenoPheno analyses (Mulcare et al. 2004; Itan et al. 2010) using interpolated allele and phenotype frequency distributions (supplementary fig. 2, Supplementary Material online) identify only small regions of India where the difference between genotypic and phenotypic frequency remains significant after taking into account sampling and phenotype measurement errors.

Lactase Persistence Genotypes and Pastoralism in India

Several other mutations in the region surrounding -13910 C>T have been found in populations with a tradition of dairying, suggesting repeated convergent evolution of the lactase persistence phenotype. We therefore included five groups in our sample—Ror, Toda, Jat Muslim, Gawli, and Yadava—to represent traditional pastoralist populations of India as we expected these to be the most likely populations to harbor previously unknown mutations. However, even in these populations, -13910 C>T is the main segregating polymorphism, with other variants making relatively small contributions to the diversity in this genomic region. We cannot, however, exclude that these groups have additional alleles in regulatory regions outside the region sequenced. Although at a countrywide level pastoralism is significantly associated with elevated frequencies of -13910*T (Wilcoxon rank sum $P = 0.0065$), the number of pastoralist groups included in our calculation is too small for the test to have strong explanatory power. We note that three of these populations (Ror, Jat Muslim, and Toda) have the first, third, and fourth highest frequencies of the -13910*T allele in the sample; these values are greater than the overall sample mean by at least 2 SD (supplementary table 1, Supplementary Material online).

We therefore examined whether these three clear outliers with much higher -13910*T frequencies could be behind the observed deviations from Hardy–Weinberg expectations in the combined sample (table 1). Indeed, following the removal of all pastoralist individuals from the pooled data sets, HWE *P* values become nonsignificant in west and south India and Indo-European and Dravidian speakers (table 2), although once again when all India is treated as a single group, disequilibrium remains highly significant (exact $P = 3.60 \times 10^{-5}$). More interestingly,

excluding all pastoralist groups from our Kruskal–Wallis tests for nonrandom associations of geographic and linguistic factors on allele frequency levels reveals that geography ($P = 3.65 \times 10^{-6}$) is slightly more strongly associated with population frequencies of -13910 C>T than language ($P = 4.36 \times 10^{-6}$).

Origins of Indian Lactase Persistence and Evidence for Positive Selection

In light of other instances of convergent evolution of the lactase persistence phenotype in human populations, a key question is whether the Indian -13910*T allele is identical by descent to the European one or the product of convergent evolution. To this end, we examined a 60 kb 15-SNP-long core region (supplementary table 2, Supplementary Material online) and identified 21 distinct haplotypes in 705 individuals of western Eurasian ancestry for whom we have genome-wide SNP data (table 3, fig. 2). This sample includes 506 unrelated individuals from Europe, Pakistan, and the Middle East taken from the HGDP-CEPH and 199 Indian individuals from our own sample of 41 populations (supplementary table 1, Supplementary Material online), none of which have a history of pastoralism. All but four of the identified haplotypes have overall sample frequency <0.05; the remaining ones can be easily mapped to haplotypes A*T, A, B, and C as previously defined (Hollox et al. 2001; Poulter et al. 2003). All -13910*T alleles in our southern Asian sample (fig. 2) are found on the previously defined European A haplotype background, strongly arguing for a single common origin. Our network clearly recapitulates the previously established relationships between, and distribution of, the high frequency haplotypes, and most of the uncommon haplotypes can be easily identified as recombinants of high frequency ones.

The -13910*T allele has been identified as having undergone strong and recent positive selection in European populations (Bersaglieri et al. 2004). We searched for evidence of selection in our Indian sample by calculating the extended haplotype homozygosity score (EHH; Sabeti et al. 2002) in a 5-Mb region surrounding the -13910 C>T site. Figure 3A–D plots the decay of EHH against genetic distance from the 15-SNP core in all four continental population groups under study; results are shown only for the four observed high-frequency haplotypes. The -13910*T associated haplotype (shown in purple in fig. 3) consistently exhibits markedly greater EHH on both sides of the 15-SNP core than any other haplotype. Similarly, REHH scores associated with the haplotype are markedly greater than those associated with any other haplotype (supplementary fig. 3, Supplementary Material online). High homozygosity encompasses both the *LCT* and the *MCM6* genes and extends nearly 1 Mb on the *LCT* side (i.e., toward the centromere), although it drops off quickly in the other direction in all groups under study, a finding consistent with previous reports (Poulter et al. 2003; Bersaglieri et al. 2004; Voight et al. 2006; Sabeti et al. 2007). The structure of this extended haplotype block is fundamentally the same in all populations as a joint analysis of all samples yields a single

Table 3. Haplotype Frequencies by Region.

Haplotype ^a	India			Europe (156)	Near East (160)	Pakistan (190)	Overall (705)
	Indo-European Speakers (138)	Non-Indo-European Speakers (61)	All India ^b (199)				
1 (A* <i>T</i>)	0.149	0.049	0.118	0.343	0.069	0.232	0.187
2 (A)	0.239	0.295	0.256	0.147	0.200	0.184	0.200
3	0.004	0.008	0.005				0.001
4					0.003	0.003	0.001
5	0.025		0.018	0.006		0.024	0.013
6	0.014	0.033	0.020	0.038	0.025	0.016	0.024
7				0.003	0.003		0.001
8	0.007	0.008	0.008				0.002
9					0.003		0.001
10					0.003		0.001
11				0.003			0.001
12 (B)	0.217	0.148	0.196	0.285	0.384	0.234	0.269
13	0.022	0.016	0.020		0.003	0.005	0.008
14					0.003	0.003	0.001
15	0.011		0.008			0.005	0.004
16 (C)	0.304	0.418	0.339	0.170	0.288	0.258	0.268
17					0.003		0.001
18	0.007	0.025	0.013			0.011	0.006
19						0.005	0.001
20					0.009		0.002
21				0.003	0.003	0.021	0.007

NOTE.—Sample sizes are shown in parentheses; italicized cells are haplotypes found in a single individual and thus likely recombinants. Haplotype numbering corresponds to figure 2.

^a Haplotype names from Hollox et al. (2001) are given in parentheses.

^b All India is the sum of Indo-European and non Indo-European speakers.

long-range haplotype associated with the T allele, with EHH scores that fall between those observed in the different continental groups (supplementary fig. 4, Supplementary Material online).

However, although the >1-Mb haplotype is found in all -13910*T-carrying populations, the decay patterns are not identical across populations, with the Indian samples exhibiting a markedly ladder-like pattern of decay, whereas Europe, the Near East, and Pakistan are all characterized by a single steep drop of EHH roughly 800 kb from the core region at around position 135.5 Mb on chromosome 2 (NCBI human genome build 36). Also striking is a clearly visible 500-kb-long region of relatively high EHH associated with haplotype B in all populations. This region is centered around 136 Mb, where two genes, *ZRANB3* and *R3HDM1*, are located. Although the first gene is a zinc-finger protein of unknown function, this second gene, which encodes an R3H domain-containing protein, has been implicated in food conversion efficiency in cattle (Barendse et al. 2007). *R3HDM1* also shows some evidence of having come under positive selection during the domestication process (Gibbs et al. 2009), suggesting that there may be other nearby functional variants under selection in humans. It is interesting to note that Tishkoff et al. (2007) similarly detected evidence of a secondary sweep centered around *R3HDM1*, and it is possible that the ancestral haplotype for -14010*C on which Tishkoff et al. base their observations may be closely related to the one we are detecting.

With the exception of haplotype A, which shows markedly greater conservation in the proximal direction in the Near Eastern sample than in any other region,

the remaining haplotypes display similar degrees of decay across populations. Finally, because Indo-European-speaking groups were repeatedly identified as being significantly associated with lactase persistence in our data, we examined EHH in Indo-European speakers alone (supplementary fig. 5, Supplementary Material online). There is a sizable overrepresentation of Indo-Europeans ($n = 138$) in our genomic Indian data set ($n = 199$), which explains the similar decay patterns in figure 3A and supplementary figure 3a (see Supplementary Material online). Of the 122 non-Indo-European chromosomes in our genome-wide data set, only 6 carry the -13910*T allele, and thus, we did not examine them independently.

Discussion

The populations of Europe, Arabia, and parts of Africa that adopted subsistence strategies that included dairying have high frequencies of individuals who can digest lactose after weaning. This phenotype has been shown to be associated with multiple functional mutations in an *LCT* enhancer region, roughly 14-kb upstream from the transcription start site (reviewed in Ingram, Mulcare, et al. 2009). At the genetic level, lactase persistence has evolved independently at least four times (Enattah et al. 2002; Tishkoff et al. 2007; Enattah et al. 2008); in two of these cases (-13910*T and -14010*C), the alleles have demonstrably come under the action of positive selection (Bersaglieri et al. 2004; Coelho et al. 2005; Tishkoff et al. 2007; Enattah et al. 2008; Itan et al. 2009). Therefore, the appearance and subsequent expansion of an agropastoral subsistence strategy

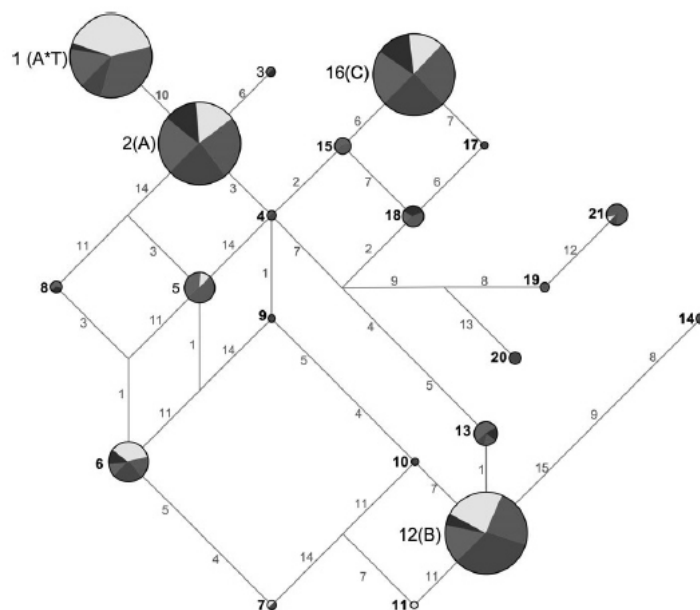


FIG. 2. Maximum parsimony neighbor joining network of 21 15-SNP-long haplotypes identified in 705 samples of western Eurasian origin. Node nomenclature corresponds to that in table 3, mutations correspond to those in supplementary table 2 (Supplementary Material online). Nodes are proportional to haplotype frequency; geographic distribution of each haplotype is coded by color: Yellow, Europe; red: Near East; blue: Pakistan; light green: Indo-European speakers from India; and dark green: non-Indo-European speakers from India.

over much of India during the last 7,000 years (Meadow 1993; Loftus et al. 1994; Possehl 1997; Fuller 2003) lead to expectations of a high frequency of lactase persistence in the subcontinent. Indeed, previous studies have suggested that lactase persistence is a common phenotype in Pakistan and northern India (Desai et al. 1970; Swaminathan et al. 1970; Gupta et al. 1971; Reddy and Pershad 1972; Bersaglieri et al. 2004; Babu et al. 2010). However, the genetic basis of adult lactase persistence in India has remained largely unexplored, leaving major questions about the origins, diversity, and history of this major cultural and nutritional adaptation unanswered. We have conducted a systematic search by sequencing the region in which the key mutations known to cause lactase persistence are found, as well as looking for new mutations, in over 2,284 individuals from 106 populations living in the Indian subcontinent.

-13910*T allele frequency predicts lactase persistence with high accuracy in most of Europe, especially in the northwestern half of the continent (Itan et al. 2010). The first study of its incidence in India showed that, at least in two mixed urban populations, this mutation occurs at frequencies > 30% (Babu et al. 2010). The results presented herein not only confirm the widespread presence of the -13910*T allele in India but also the absence of other alleles in the genomic region under study that could make significant contributions to lactase persistence frequencies. Population-level -13910*T allele frequencies within our samples range from 0 to 0.489 and predicted phenotype frequencies from 0 to 0.739. Nonetheless, the

predicted countrywide phenotype frequency in our sample, obtained from pooling all data points, is substantially lower (0.196) than estimated in previous interpolation-based studies with considerably less genetic data (~0.40) (Ingram, Mulcare, et al. 2009; Itan et al. 2010). GenoPheno analyses (Mulcare et al. 2004; Itan et al. 2010) on interpolated phenotype and allele frequencies indicate that with the exception of some small regions, this difference is not statistically significant. It should, however, be noted that there is a paucity of phenotypic data.

Short- and long-range haplotype data point strongly toward a single shared origin of the -13910 C>T mutation in Europe and India (fig. 3). Given the distribution of the -13910*T allele, the observed degree of haplotype conservation indicates a recent origin followed by a rapid rise in frequency, most likely driven by strong positive selection. There are three a priori scenarios that could explain this sharing: 1) The LRH originated somewhere in south Asia and spread westward; 2) the LRH originated in Europe and spread to India either through the Near East or the Central Asian steppe; and 3) the initial sweep that led to the establishment of the LRH occurred neither in Europe nor India but somewhere in between, either in the Central Asian steppe or in the Fertile Crescent, and spread both east and west from there. It is commonly held that -13910*T and its associated phenotype only became truly advantageous following the domestication of cattle and the adoption of a dairying culture (Simoons 1970a; McCracken

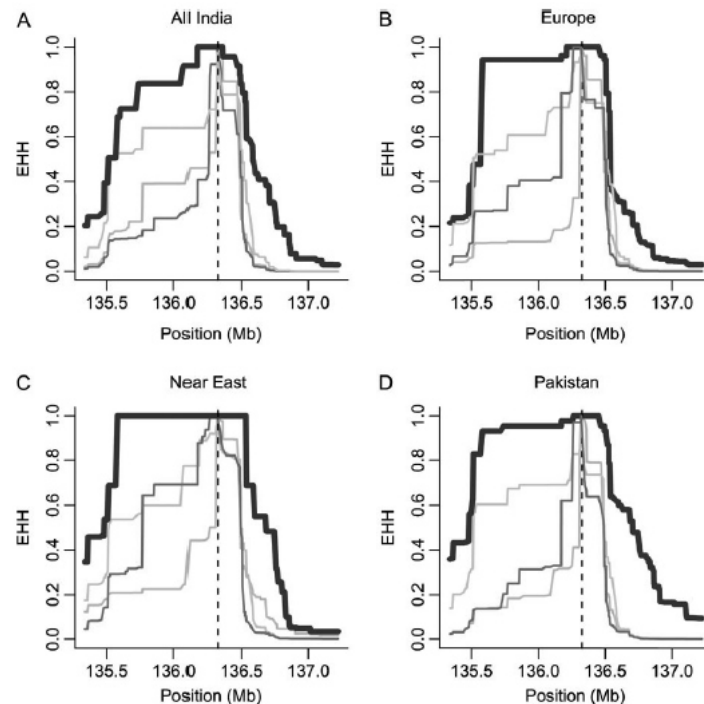


FIG. 3. EHH decay at the continental level. Haplotypes are coded by color; names correspond to those in table 3 and figure 2: purple, haplotype 1 (A**T*), associated with lactase persistence; orange: haplotype 2 (A); pale blue: haplotype 12 (B); and gray: haplotype 16 (C). The dashed line marks the location of the 15-SNP core relative to which EHH scores were calculated; it spans hg18 positions 136,278,205–136,339,424. LCT itself is located at positions 136,261,885–136,311,220, whereas MCM6 is at chr2: 136,313,666–136,350,481. The centromere is located at roughly 95 Mb; the telomere at 242 Mb. The genomic coordinates are given by Human Genome Build 36/hg18.

1971; Durham 1991). Comparison of the European and Indian history of cattle keeping suggests that the context for the selective sweep was in place in Europe roughly 2,000 years earlier than in south Asia (Copley et al. 2003; Copley, Berstan, Dudd, et al. 2005; Copley, Berstan, Mukherjee, et al. 2005; Evershed et al. 2008; Balaesque et al. 2010). The earliest evidence of cattle herding in south Asia comes from the Indus River Valley site of Mehrgarh and is dated to ~7,000 YBP (Meadow 1993), thus postdating the predicted start of the European Neolithic sweep ~7,500 YBP (Burger et al. 2007; Itan et al. 2009). It is therefore plausible that from Europe, the allele subsequently spread into Central Asia, the Near East, Pakistan, and India, although not necessarily by the same route or the same demographic event.

If the -13910**T* allele was introduced into south Asia from the west, the question arises as to whether this introduction was part of a major migratory event or a minor gene flow event potentially followed by a selective sweep within the subcontinent. A recent study has argued for a sizeable contribution from western Eurasia to the ancestral north Indian gene pool (Reich et al. 2009), although it has not provided a date or a precise origin location for this event, and the observation is compatible with low contin-

uous gene flow over a long time period. However, there is a paucity of mitochondrial DNA (mtDNA) and Y chromosome lineages in the Indian gene pool that have been assigned a Central Asian origin (Metspalu et al. 2004; Sahoo et al. 2006), suggesting that the west Eurasian genetic contribution identified by Reich et al. (2009) principally reflects gene flow from Iran and the Middle East. It is therefore likely that the -13910**T* allele was introduced to the subcontinent via a small number of migrants in the wake of the well-documented sweep for lactase persistence in the European middle Neolithic (Bersaglieri et al. 2004; Burger et al. 2007; Ingram, Mulcare, et al. 2009; Itan et al. 2009). Interestingly, a similar small-scale introgression event may also explain the presence of the 13910**T* allele in central African populations (Mulcare et al. 2004; Coelho et al. 2005). The overall low frequency of the -13910**T* allele in the subcontinent, and its absence from roughly a third of the population samples, suggests that the selective advantage it confers has been largely restricted to pastoralist groups. Three of the five sampled pastoralist populations have -13910**T* allele frequencies vastly greater than the sample mean; of those, the Ror of Haryana are responsible for the frequency peaks visible in figure 1, a pattern suggestive of

adaptation to a lifestyle tightly associated with the consumption of milk products by these populations. A similar high-frequency peak associated with the Toda in south India, however, is obscured by the presence in the sample of three nearby populations with very low -13910*T frequencies. Further studies with more pastoralist populations would be required to explore the link between the lactase persistence allele frequencies and lifestyle in India.

In the case of position -13915, a different substitution, T>G, has been described in East African and Saudi Arabian populations (Ingram et al. 2007; Tishkoff et al. 2007; Enattah et al. 2008) in association with lactase persistence, suggesting that -13915*C, as observed in India, may also be causative of the lactase persistence phenotype, whereas -13779 G>C has not been associated with any known binding sites in the *LCT* promoter region to date (Troelsen et al. 2003; Lewinsky et al. 2005). In published data, the -13779*C allele has been reported only in a single lactose nondigester Somali individual (Ingram, Raga, et al. 2009), although this is not sufficient evidence to rule out an association with lactase persistence. On a broader scale, five of the seven additional variants we have identified surrounding -13910 C>T can be found in southern India, often segregating in multiple groups. Of these groups, the pastoralist Toda are somewhat exceptional as three alleles can be found in the population at appreciable frequency: -13910*T, -13915*C, and -13779*C. Together these findings are suggestive of the beginnings of a soft selective sweep, in a manner similar to that which has been proposed in a Somali cohort where multiple potentially causative mutations occur together in lactose digesters (Ingram, Raga, et al. 2009), although further study of populations in the region and their extended haplotypes would be necessary to confirm this.

These results further indicate that, as in European populations, an association with dairying is the major selective force behind the distribution of lactase persistence in the subcontinent. We nevertheless predict phenotypic frequencies of up to 0.40 in some Indian populations with no traditional links to pastoralism, posing the question of what factors have shaped the countrywide distribution of -13910*T alleles in India among the majority of populations who do not seem to traditionally base their subsistence on dairying (Simoons 1970b). Populations from north or west India that speak Indo-European languages have, in general, substantially higher frequencies of the -13910*T allele than Dravidian speakers from the south. However, the polymorphism is clearly not confined to Indo-European speakers, and within the Indo-European speaking groups, there is a clear west to east frequency decline, whereas there are no abrupt frequency differences between geographically adjacent Indo-European and Dravidian speakers (fig. 1, supplementary table 1, Supplementary Material online). Furthermore, partial Mantel tests showed that this association disappears when geography is controlled for. This geographically contiguous patterning of variation in the locus is consistent with other genetic data from both autosomal and uniparentally inherited marker loci in India (Kivisild et al. 2003; Metspalu et al.

2004; Sahoo et al. 2006; Reich et al. 2009). A particularly striking example of the complexity of the interaction between subsistence strategy, cultural norms, and geographical proximity is found in the Nilgiri Hills, home to the pastoralist Toda, and three additional nonpastoralist groups, who have allelic frequencies ranging from 0.012 to 0.233. When considering socially defined population structure (i.e., castes and tribes), allele frequency patterns within the same endogamous caste can fluctuate vastly across geographic regions. Likewise, some Indo-European and Dravidian tribal communities, like the Bhil, Badaga, or the Toda, have higher allele frequencies than some Indo-European or Dravidian castes.

The pattern observed among speakers of Tibeto-Burman and Austroasiatic languages requires a separate explanation. In both of these groups, the observed -13910*T allele frequencies are close to 0, consistent with the fact that none of the populations studied from among these language families have known histories of pastoralism or significant milk consumption (Simoons 1970b). Here, we do find a clear-cut division between linguistic affiliation and genotype. In the case of Tibeto-Burman groups, this is again in line with previous mtDNA, Y-chromosome, and autosomal studies that identified these populations as recent migrants from eastern Asia (Metspalu et al. 2004; Sahoo et al. 2006; Reich et al. 2009), whereas the rare instances of lactase persistence in Austroasiatic-speaking populations, like the Mahali of Jharkhand, are probably due to recent gene flow from neighboring Indo-European or Dravidian speakers.

Our study has, for the first time, documented the distribution of known alleles in the *LCT* regulatory region across India, as well as the existence of previously unknown alleles. Taken together, our results indicate that the -13910*T allele is responsible for the substantial proportion of lactase persistence in the country. Furthermore, haplotype analyses indicate that the -13910*T allele in India is identical by descent to that found in Europe and western Asia, whereas examination of the pattern of haplotype block structure in the context of the archaeological history of herding across this intercontinental region suggests that the -13910*T allele was introduced to India from the west. However, within India, the lactase persistence phenotype has had a more structured adaptive history, with higher frequencies clustered in those groups that traditionally practice a dairying economy. Lactase persistence remains one of, if not, the best examples of coevolution between cultural and biological innovations, and the historical and socioeconomic complexity of India provides a unique opportunity for exploring the processes that generate human diversity.

Supplementary Material

Supplementary figures 1–5 and tables 1–5 are available at *Molecular Biology and Evolution* online (<http://www.mbe.oxfordjournals.org/>).

Acknowledgments

We are very grateful to all the individuals who donated samples for this work and to Dr V. R. Rao and Dr Madhu Bala Sharma at the Anthropological Survey of India and G. Arun Kumar for their collaboration with this work. Dr Jakka Parthasarathy and Anurag Kadian aided in the collection of pastoralist samples. We thank Mari Nelis, Georgi Hudjashov, and Viljo Soo for conducting the autosomal genotyping. This work was supported by the UK-India Education Research Initiative (grant number RG47772), the European Commission Seventh Framework Programme Internal Training Networks (LeCHE, grant number 215362-2), the European Union Seventh Framework Programme Ecogene (grant number 205419), the European Union Regional Development Fund through a Centre of Excellence in Genomics award, and a Bhatnagar Fellowship from Council of Scientific and Industrial Research, Government of India. Web resources: GLAD database: <http://www.ucl.ac.uk/mace-lab/resources/glad>; NETWORK: <http://www.fluxus-engineering.com/sharenet.htm>; and OMIM: <http://www.ncbi.nlm.nih.gov/omim>

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The -14010^*C variant associated with lactase persistence is located between an Oct-1 and HNF1 α binding site and increases lactase promoter activity

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Received: 2 December 2010 / Accepted: 3 February 2011
© Springer-Verlag 2011

Abstract In most people worldwide intestinal lactase expression declines in childhood. In many others, particularly in Europeans, lactase expression persists into adult life. The lactase persistence phenotype is in Europe associated with the -13910^*T single nucleotide variant located 13,910 bp upstream the lactase gene in an enhancer region that affects lactase promoter activity. This variant falls in an Oct-1 binding site and shows greater Oct-1 binding than the ancestral variant and increases enhancer activity. Several other variants have been identified very close to the -13910 position, which are associated with lactase persistence in the Middle East and Africa. One of them, the -14010^*C , is associated with lactase persistence in Africa. Here we show by deletion analysis that the -14010 position is located in a 144 bp region that reduces the enhancer activity. In transfections the -14010^*C allele shows a stronger enhancer effect than the ancestral -14010^*G allele. Binding sites for Oct-1 and HNF1 α surrounding the -14010 position were identified by gel shift assays, which

indicated that -14010^*C has greater binding affinity to Oct-1 than -14010^*G .

Abbreviations

<i>LCT</i>	Lactase gene
LPH	Lactase-phlorizin hydrolase
LP	Lactase persistence
LNP	Lactase non-persistence
<i>MCM6</i>	Minichromosome maintenance 6 gene
SNP	Single nucleotide polymorphism

Introduction

Persistent expression of human lactase-phlorizin hydrolase (LPH) in the small intestine is caused by allelic differences in a *cis*-acting mechanism that affects the transcription of the lactase (*LCT*) gene (Harvey et al. 1998; Wang et al. 1995). Lactase persistence (LP) can be considered as a bypass of the transcriptional down-regulation of the *LCT* gene (Fajardo et al. 1994) that normally occurs after weaning in lactase non-persistent (LNP) humans and other mammals (Lacey et al. 1994; Sebastio et al. 1989; Torp et al. 1993). The LP trait is believed to have evolved in populations where people have a long milk-drinking history. It is commonly found in the Northern European population and their descendants living elsewhere, and in pastoralist populations in the Middle East and Africa (Swallow 2003). These LP people have the ability to digest large amounts of lactose-containing products as a nutrition source throughout their life.

There have been several reports that different single nucleotide variants are associated with LP in different

Electronic supplementary material The online version of this article (doi:10.1007/s00439-011-0966-0) contains supplementary material, which is available to authorized users.

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Published online: 15 February 2011

 Springer

populations. The first one to be identified is the -13910^*T variant that co-segregates with the LP trait in Europeans (Mulcare et al. 2004). This single nucleotide polymorphism (SNP) is located 13,910 bp upstream the *LCT* gene and resides in intron 13 of the neighboring minichromosome maintenance 6 (*MCM6*) gene (Enattah et al. 2002). *MCM6* codes for a protein expressed in the intestinal crypts involved in the initiation of DNA replication, but its expression is not restricted to the small intestine like *LCT* (Barth et al. 1998; Harvey et al. 1996; Schwed et al. 2002). Subsequently other single nucleotide variants associated with the LP phenotype have been identified near to the -13910 position. One of these, a -13915^*G variant is found to be frequent in the Saudi Arabian population (Enattah et al. 2008; Imtiaz et al. 2007). It is also common in other regions of the Middle East and in people from Sudan and Ethiopia (Ingram et al. 2007, 2009b; Tishkoff et al. 2007). A -13907^*G variant has been associated with LP in Ethiopia (Ingram et al. 2009b), and is also present in Sudan, Kenya and Tanzania (Ingram et al. 2007; Tishkoff et al. 2007). A -14010^*C variant is associated with LP in Kenya and Tanzania (Tishkoff et al. 2007). This variant has recently also been found to be relatively frequent in Xhosa population in South Africa and in Ghana (Tormiainen et al. 2009). Several other rare SNPs which are likely to be associated with LP have recently also been reported (Ingram et al. 2007, 2009a, b; Khabarova et al. 2010; Lember et al. 2006; Tag et al. 2007). The different SNPs associated with LP seem to have co-evolved in different populations by positive selection from the time animal domestication was established (Ingram et al. 2009a), and at least two of the derived alleles have been shown to be associated directly with high LPH mRNA expression or LPH activity (Imtiaz et al. 2007; Kuokkanen et al. 2003; Poulter et al. 2003; Rasinpera et al. 2005). The derived variants mostly occur on distinct haplotypes, except for the -13907^*G which is found on the same haplotype as the -13910^*T (Enattah et al. 2008; Ingram et al. 2007, 2009a; Tishkoff et al. 2007). The extended haplotype backgrounds, which have been demonstrated for the -13910^*T and the -14010^*C variants, are a sign of recent positive selection (Bersaglieri et al. 2004; Ingram et al. 2009a; Poulter et al. 2003; Tishkoff et al. 2007).

LPH expression is regulated in a complex spatial and temporal pattern at the transcriptional level. It has been shown that the intestinal specific transcription factors CDX2, HNF1 α and GATA-4/6 bind upstream of the TATA-box in the *LCT* promoter and cooperatively stimulate transcription (Fang et al. 2000, 2001; Fitzgerald et al. 1998; Krasinski et al. 2001; Troelsen et al. 1992, 1997; van Wering et al. 2002, 2004). The region around -13910 position has been found to function as an enhancer

stimulating the *LCT* promoter activity (Olds and Sibley 2003; Troelsen et al. 2003b). The enhancer of the version with -13910^*T is twice as active as the ancestral -13910^*C variant in transfection experiments using the intestinal cell line Caco-2 (Lewinsky et al. 2005; Troelsen et al. 2003b). The increased activity of the -13910^*T enhancer is associated with a stronger binding of the transcription factor Oct-1 (Lewinsky et al. 2005). In addition to the Oct-1 binding, it has been found that the enhancer activity is also dependent on binding of GATA-6, a FOX-related factor and HNF4 α (Lewinsky et al. 2005).

In the present study we have analysed the gene-regulatory effect of the region containing the African -14010^*C variant associated with LP. We show that the -14010^*C variant increases the enhancer activity compared with the ancestral -14010^*G allele resulting in a higher reporter gene expression. An Oct-1 and an HNF1 α binding site were found to flank the -14010 position and the G>C nucleotide seems to increase Oct-1 binding to the region.

Materials and methods

Cell culture

The human Caco-2 cell line was cultured in minimal essential media with glutamax, supplemented with 10% foetal calf serum, 100 U/ml penicillin and 100 μ g/ml streptomycin. Cultures were grown in humidity at 5% CO₂ in 37°C. The cells were split at 80% confluence.

DNase I footprint

Nuclear extracts were prepared from differentiated Caco-2 cells (Troelsen et al. 2003a). A mixture of 150 μ g differentiated Caco-2 nuclear extract, 20 μ l gel shift buffer (25 mM Tris-HCL (pH 7.5); 5 mM MgCl₂; 60 mM KCl; 0.5 mM EDTA; 5% (v/v) Ficoll 400; 2.5% (v/v) glycerol; adjust pH 7.5) containing 1 mM DTT, protease inhibitor cocktail (1 μ l/ml) (Sigma) and 1 μ g poly dI-dC (Amersham Biosciences) was incubated on ice for 10 min. Five nanograms of a fragment of 413 bp containing the -13910^*T variant was ³²P-labelled at the -14096 position, added to the mixture and incubated for further 15 min on ice. 150 ng DNase I (Fermentas) was thereafter added and incubated for 60 s at room temperature. The DNase I digestion was stopped by adding 300 μ l stop buffer (1% SDS, 0.33 M NaAc and 5 mM EDTA). The sample was extracted with phenol/chloroform and ethanol precipitated. The precipitated ³²P-labelled DNA was analysed by denaturing polyacrylamide gel electrophoresis (PAGE) (6% and 6 M urea) followed by autoradiography.

Gel shift assays

Probes were made by annealing complementary sense and antisense oligonucleotides with a 5' A overhang followed by end-labelling with [γ - 32 P]ATP (Amersham Biosciences) using T4 polynucleotide kinase (Fermentas). Reactions for competition experiments contained 6 μ g differentiated Caco-2 nuclear extract, 0.25 μ g poly dI-dC (Amersham Biosciences) and 2.5 pmol unlabelled competitor oligonucleotides were mixed with 3 μ l Gorski dialyse buffer [25 mM Hepes (pH 7.6); 40 mM KCl; 0.1 mM EDTA; 10% (v/v) glycerol] and 10 μ l gel shift buffer containing 1 mM DTT, protease inhibitor cocktail (1 μ l/ml) (Sigma). The mixture was pre-incubated for 10 min on ice, before 2.5 fmol probe was added and incubated for further 20 min on ice. For supershift assays, 1–2 μ l Oct-1, HNF1 α or HNF1 β antibodies (Santa Cruz Biotechnology) were added after incubation with the labelled probe. The reaction was incubated for another 15 min on ice. DNA–protein complexes were resolved in 0.5 \times TBE [44.5 mM Tris–HCL (pH 8), 44.5 mM boric acid, 1 mM EDTA (pH 8)] on 5% nondenaturing polyacrylamide gels.

Promoter/enhancer constructs and transfection

The primer sequences used to PCR-amplify the different lengths of the enhancer regions are shown in Table 1 of the supplementary material. The primers were designed to add an upstream 5' *Bam*HI and a downstream 3' *Sal*I restriction site to the amplified products.

The three non-overlapping fragments of the 450 bp *LCT* enhancer region (–14,133 bp to –13,684 bp) were generated by PCR-amplification of the pGL3hLPH1085–13910*T or the pGL3hLPH1085–13910*C enhancer construct (Troelsen et al. 2003b), to generate a 131 bp of the 5' region, a 170 bp of the core region containing either version of the –13910 variant and a 149 bp of the 3' region.

The full length –14010*C variant of the 450 bp region was generated by site-directed mutagenesis in a two-step PCR-amplification. The –14010*C mutation was introduced in the 450 bp enhancer using two overlapping primers containing the –14010*C variant. The region was amplified in two separate PCRs to generate a left and a right fragment using the pGL3hLPH1085–13910*C enhancer construct (Troelsen et al. 2003b), as template in the first PCR step. The two fragments were then annealed to create an overlap at –14010*C, and used as template in the second PCR, to amplify the whole 450 bp fragment including both restriction sites at the end.

The 5' deletion of the 450 bp region was performed by PCR-amplification using the pGL3hLPH1085 construct containing either the African LP –14010*C variant, the European LP –13910*T variant or the ancestral

–13910*C variant of the 450 bp enhancer region as templates. All amplified products were TA-cloned into the pCR 2.1 plasmid (Invitrogen). The fragments were inserted into the pGL3hLPH1085 plasmid via *Bam*HI and *Sal*I digestion, and sequences were verified. For transient transfection, Caco-2 cells were grown to 80% confluence and seed in 24-well plates containing 5×10^4 Caco-2 cells per well and plated the day prior to transfections. The cells were transfected using 5 μ l (5.47 mmol) Exgen 500 (Fermentas) with a total of 0.3 μ g DNA per well by adding 0.05 μ g reporter gene plasmid, 0.025 μ g pCMV-LacZ plasmid (Promega) and 0.225 μ g pBluescript SK⁺ plasmid (Stratagene). The cells were harvested 2 days after transfection and measured for luciferase and β -galactosidase activity using the Dual-Light[®] luminescent reporter gene assay (Applied Biosystems). A Student unpaired *t* test was used to test for significance between the transfection results.

Results

Functional characterization of the human *LCT* enhancer

The *LCT* enhancer activity has previously been characterized by transfection experiments using a 450 bp fragment (Lewinsky et al. 2005; Troelsen et al. 2003b). In order to refine the location of the enhancer activity we first divided the 450 bp region (–14133 to –13684) into three smaller non-overlapping fragments to generate a 5' region of 131 bp (–14133 to –14003) that contains the –14010 position, a core region of 170 bp (–14002 to –13833) that includes the –13910 position and a 3' region of 149 bp (–13832 to –13684). These fragments were cloned at a distance upstream (approximately 3 Kb) of the 1,085 bp *LCT* promoter in a luciferase reporter gene plasmid, transfected into the intestinal cell line Caco-2 and compared to the full-length 450 bp enhancer construct (Fig. 1). The 5' and 3' regions of the enhancer had no effect on the reporter gene expression. The core region containing either version of the –13910 variant caused an ~2 to 3-fold decrease of the reporter gene compared with the full-length enhancer construct. Thus the 450 bp region that resides outside the 170 bp core region influence the enhancer activity. The reduced enhancer function of the 170 bp core region contains the previously identified binding sites for Oct-1 at the –13910 position, GATA, a potential related FOX and HNF4 α . It has previously been shown that these binding sites are each essential for the enhancer to function (Lewinsky et al. 2005).

A stepwise 5' deletion analysis of the 450 bp enhancer region from –14,133 to –13,910 bp was performed to identify functional gene-regulatory sites. Caco-2 cells were

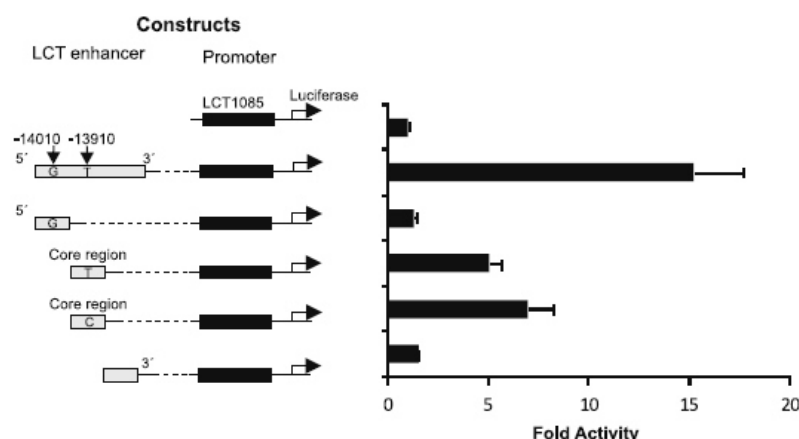


Fig. 1 Deletion analysis of the *LCT* enhancer. The 450 bp *LCT* enhancer of the European LP -13910^*T variant (-14010^*G ; -13910^*T) or the ancestral variant (-14010^*G ; -13910^*C) were divided into three non-overlapping regions: a 5' region containing the -14010^*G (131 bp), a core region (170 bp) containing either version of the $-13910^*C/T$ variant and a 3' region (149 bp). These were cloned at a distance upstream the 1,085 bp human *LCT* promoter (LCT1085) in the pGL3 luciferase reporter gene plasmid. The

reporter gene plasmids were transiently transfected into the Caco-2 cell line and analysed for luciferase and beta-galactosidase activity after 2 days. The luciferase activities were corrected for transfection efficiency using the beta-galactosidase activities, and normalised to the activity of LCT1085. The relative activities are given as mean \pm SD depicted as bars ($n = 4$). All *LCT* enhancer constructs showed significantly higher luciferase activities compared with the *LCT* promoter construct test with a Student *t* test ($P < 0.05$)

transfected with luciferase reporter gene constructs containing either the *LCT* promoter alone or the *LCT* promoter with deleted enhancer fragments using either the African LP -14010^*C variant (-14010^*C ; -13910^*C) or the European LP -13910^*T variant (-14010^*G ; -13910^*T), or the ancestral variants (-14010^*G ; -13910^*C) (Fig. 2). The full-length enhancers containing the -14010^*C or the -13910^*T variant had a ~ 1.5 -fold higher activity compared with the ancestral variant, which is in agreement with previous reports (Lewinsky et al. 2005; Olds and Sibley 2003; Tishkoff et al. 2007; Troelsen et al. 2003b). Deletion of the region from $-14,133$ to $-14,020$ bp increases the enhancer activity of all three versions to the same extent. This indicates that gene-regulatory site(s) located upstream the -14020 position reduce the enhancer activity. The enhancer activity did not change significantly when deleting the region up to $-14,000$ bp. But a further deletion of 10 nucleotides up to $-13,990$ bp increased the enhancer ~ 3 to 4-fold compared with the full-length enhancer. These results show that the first 144 bp ($-14,133$ to $-13,990$) of the 5' region reduce the enhancer function. With each further 5' deletion the luciferase activity is decreased showing that several important gene-regulatory sites for the enhancer activity are located downstream of -13990 . We have previously found that Oct-1 and GATA-6 bind to the region between -13940 and -13910 and that both transcription factors are necessary for the enhancer activity (Lewinsky et al. 2005), which is consistent with the effect of deletion of this region.

Identification of transcription factor binding sites around the -14010 position

In order to identify gene-regulatory elements located in the -14010 SNP region, a DNase I footprint analysis of the 5' end of the *LCT* enhancer was performed (Fig. 3). The analysis revealed a footprint (FP1) from -14021 to -14009 that includes the -14010 position and another footprint (FP2) from -14007 to -13993 in the 5' region of the enhancer.

To identify the nucleotide positions that are required for transcription factor binding in the FP1 footprint, mutated oligonucleotides (mut1-8) with a series of different three base pair mutations covering the FP1 sequence (-14030 to -14006) were analysed by gel shift assays (Fig. 4a). The FP1G probe with the ancestral -14010^*G variant generated a specific gel shift (complex I) (Fig. 4b, lane 1). Complex I formation was clearly competed by the FP1G mut1-3 (lanes 3-5) and mut7-8 oligonucleotides (lanes 9-10), whereas the FP1G mut4-6 oligonucleotides did not compete (lanes 6-8). This demonstrates that the sequence covered by the mutations in the FP1G mut4-6 oligonucleotides is important for generating complex I. This region is underlined in the FP1G sequence (FP1 site) in Fig. 4a, and corresponds to a canonical octamer motif (Sturm et al. 1988). Supershift analysis with an Oct-1 antibody confirmed that Oct-1 binds to this sequence. The Oct-1 antibody supershifted complex I in the case of the FP1C probe containing the -14010^*C variant (Fig. 4c, lane 2) but only

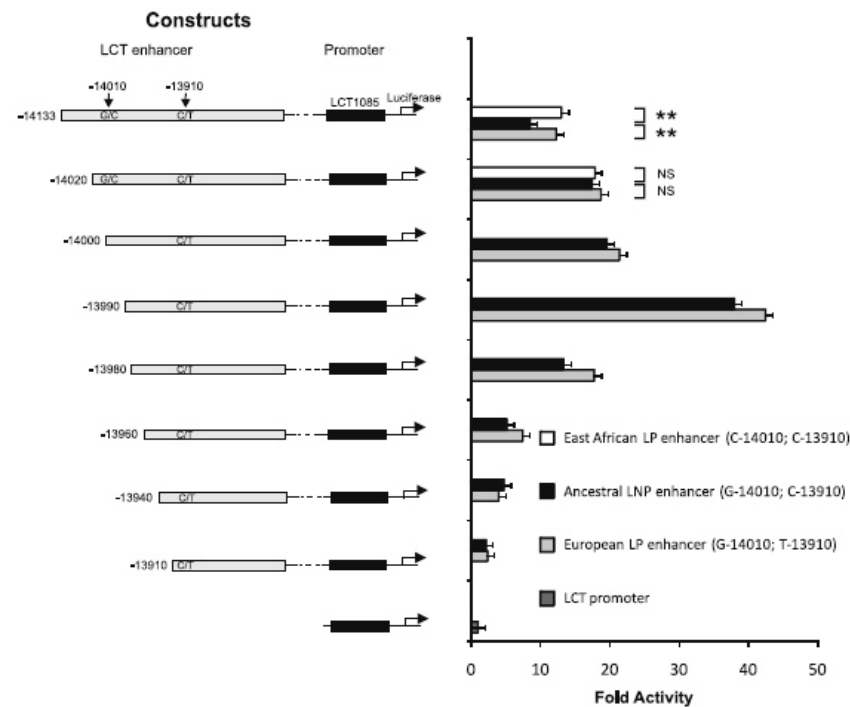


Fig. 2 5' deletion analysis of the *LCT* enhancer carrying different variants. The 450 bp *LCT* enhancer containing, respectively, the African LP -14010*C (-14010*C; -13910*C), the European LP -13910*T (-14010*G; -13910*T) or the ancestral variant (-14010*G; -13910*C) was successively deleted from -14,133 to -13,910 bp. The various deleted enhancer fragments were cloned at a distance upstream the 1,085 bp human *LCT* promoter (LCT1085) in the pGL3 luciferase reporter gene plasmid. The reporter gene plasmids were transiently transfected into the Caco-2 cell line and analysed for luciferase and beta-galactosidase activity after 2 days.

The luciferase activities were corrected for transfection efficiency using the beta-galactosidase activities, and normalised to the activity of the LCT1085. The relative activities are given as mean \pm SD depicted as bars ($n = 4$). A *t* test was used to test statistical significance of the difference between the luciferase activities of the enhancer construct containing both position -13910 and -14010 (the -14133 and the -14020 constructs). The LP enhancer variants were tested against the corresponding ancestral variant (** $P < 0.01$; NS not significant)

inhibited complex I formation in the case of the FP1G probe containing the -14010*G variant (Fig. 4c, lane 5). A sequence containing a canonical Oct-1 site previously used to investigate Oct-1 binding to the -13910 position (Lewinsky et al. 2005), was able to compete the complex I formation (lanes 5 and 10) in Fig. 4d, supporting the interpretation that Oct-1 binds to the FP1 probe containing either variants. We also compared the effect of the -14010 variants on the protein-binding affinity of complex I. No substantial difference was seen between the intensity of complex I generated by the FP1C and FP1G probes. Both oligonucleotides efficiently competed with both probes to prevent complex I formation (lanes 3–4 and 8–9) in Fig. 4d. The less intense and faster migrating complex that appeared using the FP1 probe (Fig. 4b–d) likely correspond to a CDX2 binding, since the FP1 sequence partly contains the previously identified CDX2 site at -14032 to -14022 (Lewinsky et al. 2005).

A similar approach was used to identify the factor generating the FP2 footprint and its binding site was identified using mutated oligonucleotides (mut1–8) in gel shift assays (Fig. 5a). The FP2G probe containing the ancestral -14010*G variant and covering the FP2 sequence (-14011 to -13987) has a 6 bp overlap with the FP1 sequence and generated a specific gel shift (complex II) (Fig. 5b, lane 1). Of the competitive FP2G mut1–8 oligonucleotides, the FP2G mut3 and mut5–6 oligonucleotides (lanes 5 and 7–8) were not able to compete with the protein that generated complex II in Fig. 5b. The positions of the mutations in these oligonucleotides are underlined in the FP2G sequence (FP2 site) in Fig. 5a. The binding sequence is similar to an HNF1 site, and HNF1 is therefore predicted to bind the FP2 site. HNF1 binds two seven-base half sites (on opposite DNA strands) with a fixed spacing of one base (Locker et al. 2002), which is seen with footprint FP2 (Fig. 3), and does not include the -14010 position.

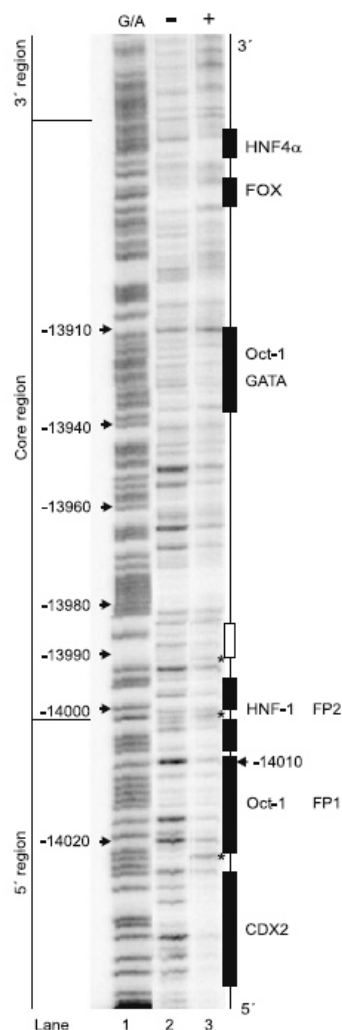


Fig. 3 DNase I footprinting of the *LCT* enhancer. Location of novel footprints (FP1 and FP2) and previous identified binding sites (HNF4 α , FOX, Oct-1, GATA and CDX2) in the *LCT* enhancer region are marked by black boxes. The footprint marked by a white box was not investigated. Lane 1: the G/A sequencing lane was used to assign the position of protected sites. Hypersensitive sites are marked by an asterisk. Lanes 2 and 3: none (–) or 150 μ g differentiated Caco-2 nuclear extract (+) was treated with DNase. The –14010 position is marked by an arrow to the right between footprint FP1 and FP2 that were further analysed. The left site of the footprint shows, respectively, the division of the 450 bp enhancer region in a 5' region of 131 bp, a core region of 170 bp and a 3' region of 149 bp (see Fig. 1) and the position of the successive 5' deletion of the enhancer region are marked by arrows (see Fig. 2) that were analysed functionally

Further gel shift and supershift assays supported the interpretation that an HNF1 site is located within position –14009 to –13993. The formation of complex II was

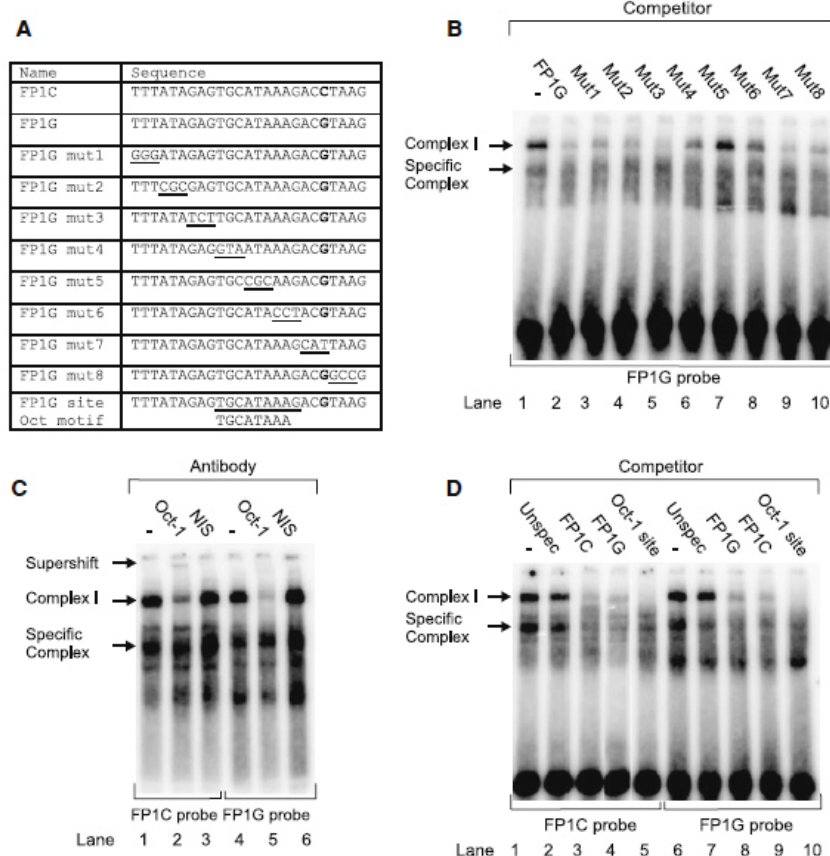
competed with a sequence containing the HNF1 site (CE-2c) from the *LCT* promoter (lanes 5 and 10) in Fig. 5c (Mitchellmore et al. 2000; Spodisberg et al. 1999). The addition of HNF1 α antibody to the binding reaction supershifted complex II using either the FP2C or the FP2G probe (lanes 2 and 7) in Fig. 5d. In contrast an HNF1 β antibody generated only a very weak supershift (lanes 3 and 8) in Fig. 5d, indicating that complex II is mainly composed of HNF1 α homodimers. This was to be expected since the applied nuclear extract was prepared from differentiated Caco-2 cells that express much more HNF1 α than HNF1 β (Boudreau et al. 2001). The factors generating the two lower bands observed using the FP2 probe were not identified (Fig. 5b–d). However, the mutation analysis showed that the CCATTT sequence is important for the factors to interact with the FP2 sequence (lanes 6–7) in Fig. 5b.

A probe (FP1/2) covering the parts of FP1 and FP2 (–14026 to –13996) that contain both the Oct-1 site in FP1 and the HNF1 α site in FP2 (Fig. 6a) was investigated for Oct-1 and HNF1 α binding by gel shift and supershift assays. The FP1/2 probes generated two specific gel shifts (complex I and II). The intensity of the complex I shift was slightly greater using the FP1/2C probe containing the –14010*C variant (lane 1, Fig. 6b) compared with the FP1/2G probe containing the –14010*G variant (lane 1, Fig. 6c). Complex I formation with the FP1/2C probe was competed more efficiently by the unlabelled FP1/2C (lanes 3, Fig. 6b) than the FP1/2G oligonucleotides (lanes 4, Fig. 6b). Complex I and complex II were affected by Oct-1 and HNF1 α antibody treatment, respectively (lanes 9–10) in Fig. 6b–c. The FP1/2C probe generated a stronger Oct-1 supershift for complex I compared with FP1/2G. HNF1 α antibody inhibited complex II formation to a similar extent for both alleles (a weak supershift was seen using the FP1/2C probe) (Fig. 6b). In addition, complex I and complex II could be competed with unlabelled FP1G and FP2G oligonucleotides, respectively (lanes 5 and 7), whereas the FP1G mut5 oligonucleotide that contains mutations that interfered with the Oct-1 binding did not compete (lane 6) in Fig. 6b–c. The FP2G mut3 oligonucleotide that involves the HNF1 α site did compete with the probes but not as effectively (lane 8) as in Fig. 6b–c.

Discussion

–14010 resides at the 5' end of a previously characterised 450 bp sequence with *LCT* enhancer function, which contains –13910*T that is associated with LP in Europeans (Lewinsky et al. 2005; Trøelsen et al. 2003b). However, the 5' end of the enhancer sequence was previously not thoroughly investigated. Here we show that the 5' region of the

Fig. 4 Gel shift and supershift assays of the FP1 region. **a** The sense strands of the oligonucleotides used in the analysis are shown. The -14010 position is in **bold** and the mutated sequences and the FP1G site are underlined. **b–d** Differentiated Caco-2 nuclear extracts were incubated with the FP1 probe containing either the ancestral -14010^*G variant (FP1G) or the LP -14010^*C variant (FP1C). Competitive oligonucleotides or antibodies or no (–) are shown at the top of each lane. The probes applied to each lane are indicated at the bottom. Complex I and the lower specific complex are marked with arrows. **b** The mutated FP1G oligonucleotides (mut1–8) were added in lanes 3–10. **c** Oct-1 antibody was added in lanes 2 and 5. Non-immune serum (NIS) was added in lanes 3 and 6. Oct-1 supershift of complex I is marked by an arrow. The free probes at the bottom of the gel were run into the buffer. **d** An unspecific (unspec) oligonucleotide was added in lanes 2 and 7. An oligonucleotide containing an Oct-1 site was added in lanes 5 and 10



LCT enhancer has a regulatory effect on the enhancer activity. Our functional analysis suggests that the -14010^*C variant associated with LP act as a stronger enhancer of the *LCT* promoter compared with the ancestral -14010^*G variant. In the 5' region of the *LCT* enhancer two binding sites were identified by footprint analysis and shown to flank the -14010 position. These were identified as being an Oct-1 (-14021 to -14009) and an HNF1 α site (-14007 to -13993) with the -14010 position being part of the Oct-1 site. Interestingly, there was no pronounced difference in Oct-1 binding to the -14010^*C and -14010^*G variants in the gel shift assays using the FP1 probes that only contain the Oct-1 site (Fig. 4). However, a slight difference in the binding to -14010^*C than to the -14010^*G variant was observed using the FP1/2 probe which contains both the Oct-1 and the HNF1 α sites (Fig. 6). This indicates that the Oct-1 binding to the -14010^*C variant is influenced by the downstream sequence that covers the HNF1 α site. The small differences in the Oct-1 binding to the -14010 allelic variants in EMSAs we have found, might be expected to be more

pronounced in adult intestine where the pattern of transcription factor expression and activity allows higher expression than in the Caco-2 cell line (Mitchellmore et al. 1998).

Thus, the fact that the -14010^*C variant increases the *LCT* enhancer activity functionally supports the notion that this variant could be the causal mutation preventing the developmental down-regulation of the *LCT* gene in some LP individuals from Africa. Interestingly, the LP -14010^*C variant is located in an Oct-1 binding site as it is the case with the European LP -13910^*T variant indicating that Oct-1 is involved in the generation of the lactase persistence phenotypes in these two cases. In addition a $T > -14009^*G$ SNP has been found in a Somali cohort (Ingram et al. 2009b) and a rare $C > -14011^*T$ variant (Lember et al. 2006). Since these SNPs are located right next to the -14010 position it is possible that these variants can also alter Oct-1 binding at position -14021 to -14109 . The $C > -13907^*G$ and $T > -13915^*G$ SNPs associated with LP located in the immediate vicinity of the European -13910^*T SNP have been shown to bind Oct-1. Gel shift assays revealed that Oct-1 binds with similar affinity to a

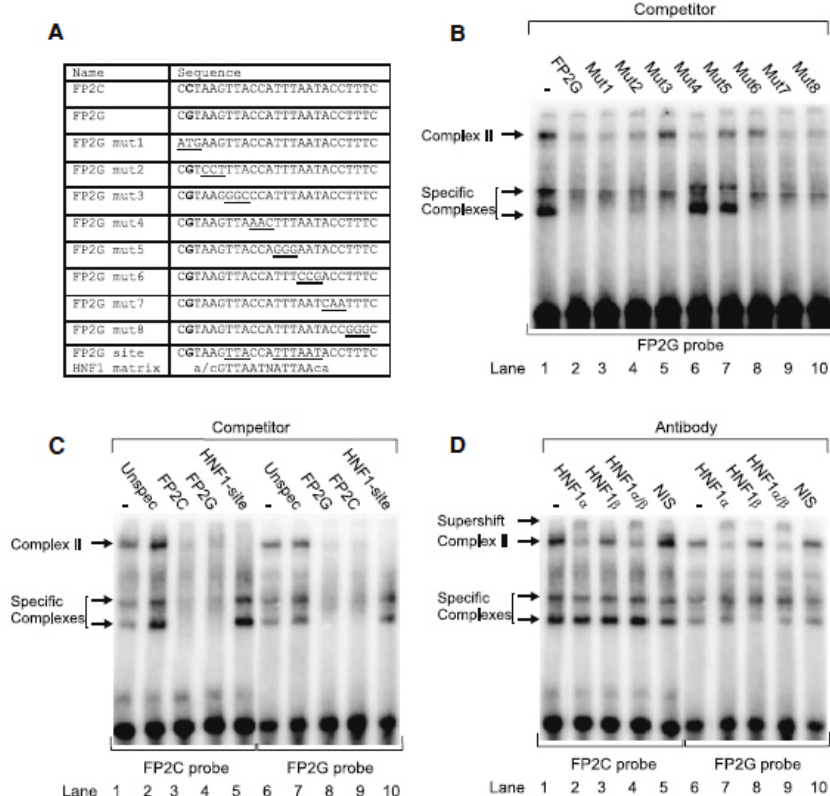


Fig. 5 Gel shift and supershift assays of the FP2 region. **a** The sense strands of the oligonucleotides used in the analysis are shown. The –14010 position is in **bold** and the mutated sequences and the FP2G site are underlined. **b–d** Differentiated Caco-2 nuclear extracts were incubated with the FP2 probe containing either the ancestral –14010*G variant (FP2G) or the LP –14010*C variant (FP2C). Competitive oligonucleotides or antibodies or no (–) are shown at the top of each lane. The probes applied to each lane are indicated at the

bottom. Complex II and the two lower specific complexes are marked with arrows. **b** The mutated FP1G oligonucleotides (mut1–8) were added in lanes 3–10. **c** An unspecific (unspec) oligonucleotide was added in lanes 2 and 7. An oligonucleotide containing an HNF1 (CE-2c) site from the *LCT* promoter was added in lanes 5 and 10. **d** HNF1 α , HNF1 β , HNF1 α/β antibodies or non-immune serum (NIS) was added in lanes 2–5 and lanes 7–10. HNF1 α supershift of complex II is marked with an arrow

sequence containing the –13907*G variant as the –13910*T variant whereas the –13915*G shows reduced Oct-1 binding in some (Enattah et al. 2008; Ingram et al. 2007) but not all studies (Olds et al. 2011). However, functional analyses of the 450 bp region containing either the –13907*G or the –13915*G variant have also shown that these two LP variants increase the enhancer activity (Enattah et al. 2008; Olds et al. 2011; Tishkoff et al. 2007). Two other rare SNPs (T > –13913*C and G > –13914*A variant) located in the Oct-1 site (–13921 to –13909) have been identified (Ingram et al. 2007; Khabarova et al. 2010; Tag et al. 2007). These two variants of the *LCT* enhancer have not yet been investigated. The presence, in an Oct-1 site, of several point mutations that are associated with the LP phenotype, suggests that Oct-1 is a key player in

modulating the developmental expression of the *LCT* gene. Oct-1 belongs to the POU family of transcription factors contains a POU-specific domain and a homeo domain. The two domains are linked together by a flexible linker that allows Oct-1 to adopt various arrangements through flexible amino acid–base interactions on the DNA strand. Therefore Oct-1 is known to be able to bind to DNA in different conformations. This provides versatility in how Oct-1 binds to the DNA strand and interacts with other gene-regulatory proteins (Herr and Cleary 1995). For this reason Oct-1 has the ability to recognize diverse DNA sequences. This is seen with the two Oct-1 sites in the 450 bp *LCT* enhancer that differ from each other. The Oct-1 site at the –14010 position contains a canonical octamer motif, whereas the Oct-1 site at the –13910 position belongs

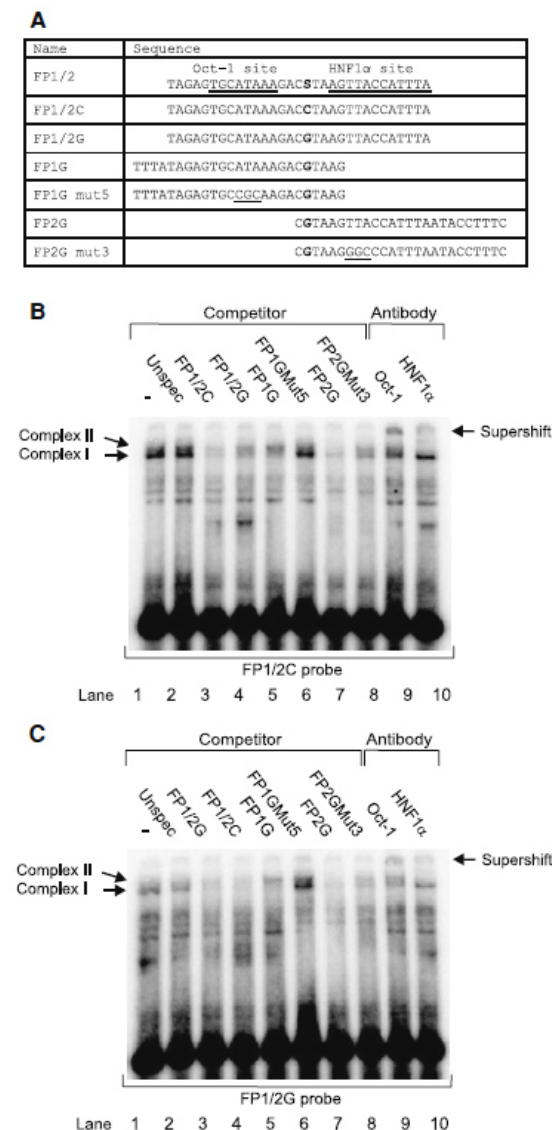


Fig. 6 Gel shift and supershift assays of the FP1/2 region. **a** The sense strands of the oligonucleotides used in the analyses are shown. The -14010 position is in **bold** and the Oct-1 and HNF1 α site and mutated sequences are underlined. **b**, **c** Differentiated Caco-2 nuclear extract was incubated with the FP1/2 probe containing either the LP -14010^*C variant (FP1/2C) (**b**) or the ancestral -14010^*G variant (FP1/2G) (**c**). Competitive oligonucleotides or antibodies or none (–) are shown at the *top* of each lane. The probes applied to each lane are indicated at the *bottom*. Complex I and complex II are marked with *arrows*. An unspecific (unspec) oligonucleotide was added in lane 2. The mutated FP1G mut5 and FP2G mut3 oligonucleotides were added in lanes 6–8. Oct-1 or HNF1 α antibodies were added in lanes 9 and 10. The position of the Oct-1 and HNF1 α supershifts are marked with an *arrow*

to the non-canonical group containing a TAAT sequence. However, like the -13910 position, -14010 is at the edge of the Oct-1 binding site.

It is remarkable that relative small change of Oct-1 binding to either the -14010 or the -13910 SNP position has such a pronounced effect on the developmental regulation of the LPH expression. Our results indicate that the developmental down-regulation of LPH is mediated through loss of enhancer activity after weaning, and that the LP phenotype can be mediated by any mutation increasing the enhancer activity to a level that will rescue the *LCT* gene from developmental inactivation.

Acknowledgments We thank Lotte Bram for excellent technical assistance. This work was supported by the Lundbeck Foundation, the Augustinus Foundation, Aase and Ejnar Danielsen Foundation, the Danish Research Council and EU FP7 ITN Grant 215362-2 (LeCHE).

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Research

Evolution of lactase persistence: an example of human niche construction

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Niche construction is the process by which organisms construct important components of their local environment in ways that introduce novel selection pressures. Lactase persistence is one of the clearest examples of niche construction in humans. Lactase is the enzyme responsible for the digestion of the milk sugar lactose and its production decreases after the weaning phase in most mammals, including most humans. Some humans, however, continue to produce lactase throughout adulthood, a trait known as lactase persistence. In European populations, a single mutation (-13910^*T) explains the distribution of the phenotype, whereas several mutations are associated with it in Africa and the Middle East. Current estimates for the age of lactase persistence-associated alleles bracket those for the origins of animal domestication and the culturally transmitted practice of dairying. We report new data on the distribution of -13910^*T and summarize genetic studies on the diversity of lactase persistence worldwide. We review relevant archaeological data and describe three simulation studies that have shed light on the evolution of this trait in Europe. These studies illustrate how genetic and archaeological information can be integrated to bring new insights to the origins and spread of lactase persistence. Finally, we discuss possible improvements to these models.

Keywords: lactase persistence; niche construction; Neolithic; domestic animals; dairying; natural selection

1. THE BIOLOGY OF LACTASE PERSISTENCE

(a) *Niche construction, lactase persistence phenotype and genotypes*

In biological evolution, natural selection acts on traits that are heritable. The vast majority of these traits are inherited by the transmission of DNA sequences from parent to offspring, i.e. genetic inheritance. But other aspects of the biology of an organism can be inherited extra-genetically, such as certain culturally transmitted behaviours [1,2] and features of the environment that have been shaped by ancestral populations [3]. Since this kind of extra-genetic inheritance can play a key role in the survival of an organism and the

evolutionary trajectory of a species, it has been assigned the term ‘niche construction’ [4,5]. A deeper understanding of the relationship between genetic evolution and niche construction can come from evolutionary theory, notably by recognizing that humans are far from unique in their ability to change their own selective environments [6]. However, because human culture has strongly modified our environments with such remarkable ecological and evolutionary consequences [2], human gene–culture coevolution provides some of the clearest and most spectacular examples of niche construction.

Gene–culture coevolutionary theory integrates cultural variation in the analysis of differential transmission of genes from one generation to the next [7]. This theory can be explicitly used to explore the evolutionary consequences of niche construction when a stable transmission of learned information is conveyed

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One contribution of 13 to a Theme Issue ‘Human niche construction’.

between successive generations [8,9]. Indeed, cultural processes can change the human selective environment and thereby affect which genotypes survive and reproduce [1,2]. If the cultural inheritance of an environment-modifying human activity persists for long enough to generate a stable selection pressure, it will be able to co-direct human evolution. There are many examples of this in human evolution [1–3,10] but none are so well studied, clear-cut, widespread and well supported as the coevolution of lactase persistence (LP) and dairying [4,11–13].

Lactose is the main carbohydrate in milk and is a major energy source for most young mammals. The enzyme responsible for hydrolysis of lactose into glucose and galactose is lactase (or lactase-phlorizin-hydrolase, LPH). Without this enzyme, mammals are unable to break down and thus use lactose, and since milk is the essential component of young mammals' diet, lactase activity is fundamental to the early development of most mammals. After the weaning period is over, lactase production usually declines, although the mechanisms and evolutionary reasons for this downregulation are not fully understood. However, some humans continue to express lactase throughout adult life, and are thus able to digest the lactose found in fresh milk. This trait is called LP.

The LP trait frequency is found in around 35 per cent of adults living in the world today [14,15], but varies widely among human populations, both between and within continents (figure 1a). High frequencies of LP are generally observed in northern European populations. Indeed, LP frequency can vary from 15–54% in eastern and southern Europe to 62–86% in central and western Europe, and to as high as 89–96% in the British Isles and Scandinavia [15,16]. In India, LP frequency is higher in the north (63%) than further south (23%) or east [17]. There are relatively little data on East Asians but it seems that the trait is rare there. In Africa, the distribution of LP is very patchy, with high frequencies being observed mainly in traditionally pastoralist populations [14,18–20]. For example, LP reaches 64 per cent in Beni Amir pastoralists (Sudan), whereas in Dounglawi (Sudan), a neighbouring non-pastoralist population, LP frequency is around 20 per cent [11,21,22].

In recent years, a number of single nucleotide polymorphisms (SNPs) have been found in association with the LP trait in different populations. The first to be identified, -13910^*T , is found not in the *LCT* gene (the lactase gene) but within an intron of a neighbouring gene, *MCM6* [23]. *In vitro* studies indicate that this nucleotide change affects lactase promoter activity [24,25] and thus is highly likely to cause LP, although it is currently not possible to exclude tight linkage disequilibrium with another, as yet unobserved, functional variant. An interpolated map showing the global distribution of -13910^*T can be seen in figure 1b, using published data [14,15,26–30] supplemented with recently collected data. A cursory comparison of figure 1a,b shows that while the -13910^*T allele may explain the distribution of LP in Europe, it cannot explain the distribution of LP in Africa or the Middle East. Indeed, Mulcare et al. [31]

showed this formally using a robust statistical framework, and other studies have identified additional LP-associated alleles that explain much of the distribution of LP in Africa [16,20,32]. Interestingly, all these variants are located within 100 nucleotides of -13910^*T in the same intron of the *MCM6* gene, a region that is functionally important for the expression of lactase *in vitro* [24,33]. Because these various LP-associated alleles are found on several different haplotypic backgrounds [14,20,34], it is now clear that LP has evolved multiple times and is thus an example of convergent evolution [35].

Using genetic variation in regions surrounding *LCT*, it is possible to obtain estimates of the age of specific LP-associated alleles. Dates of origin for -13910^*T ranging between 2188 and 20 650 years ago [36], and between 7450 and 12 300 years ago [37] have been obtained using extended haplotype homozygosity (EHH) and variation at closely linked microsatellites, respectively. Similar dates (1200–23 200 years old) were also obtained for one of the major African variants (-14010^*C) using EHH [20]. These date estimates are remarkably recent for alleles that are found at such high frequencies in multiple populations. It is easy to envisage recent alleles being rare since they change in frequency slowly, and in a directionless way, by genetic drift. However, a recent allele that has reached such high population frequencies requires more than genetic drift alone; it requires the extra 'kick' of natural selection. Indeed, the estimated selection strengths required to explain the age/frequency distributions of -13910^*T [36]—and of -14010^*C [20]—are enormous (1.4–19 and 1–15%, respectively), which are among the highest estimated for any human genes in the last approximately 30 000 years [36,38].

(b) Selection hypotheses on lactase persistence

The reasons why LP should provide such a selective advantage are still open to debate (see discussion below). However, since this trait has been mainly identified in dairying-practising or pastoralist populations [11] and since fresh milk and some milk products are the only known naturally occurring sources of lactose, it is unlikely that LP would be selected without a supply of fresh milk. Interestingly, the date estimates for the emergence of -13910^*T and -14010^*C bracket archaeological dates for the spread of domestic animals and dairying into Europe and the spread of pastoralism in Africa (south of the Sahara, into Kenya and northern Tanzania), respectively [11,20,39–42]. This supports the idea that LP coevolved with the cultural adaptation of dairying as a gene–culture coevolution process.

Nonetheless, the correlation between LP and milk consumption is not complete [11,16,31,32]. In lactase non-persistent individuals, the fermentation by colonic bacteria and osmotic effects of undigested lactose often cause symptoms such as abdominal pain, bloating, flatulence and diarrhoea. However, it has been shown that some lactase non-persistent individuals can consume lactose-containing products without any obvious ill effects. For example, the low LP

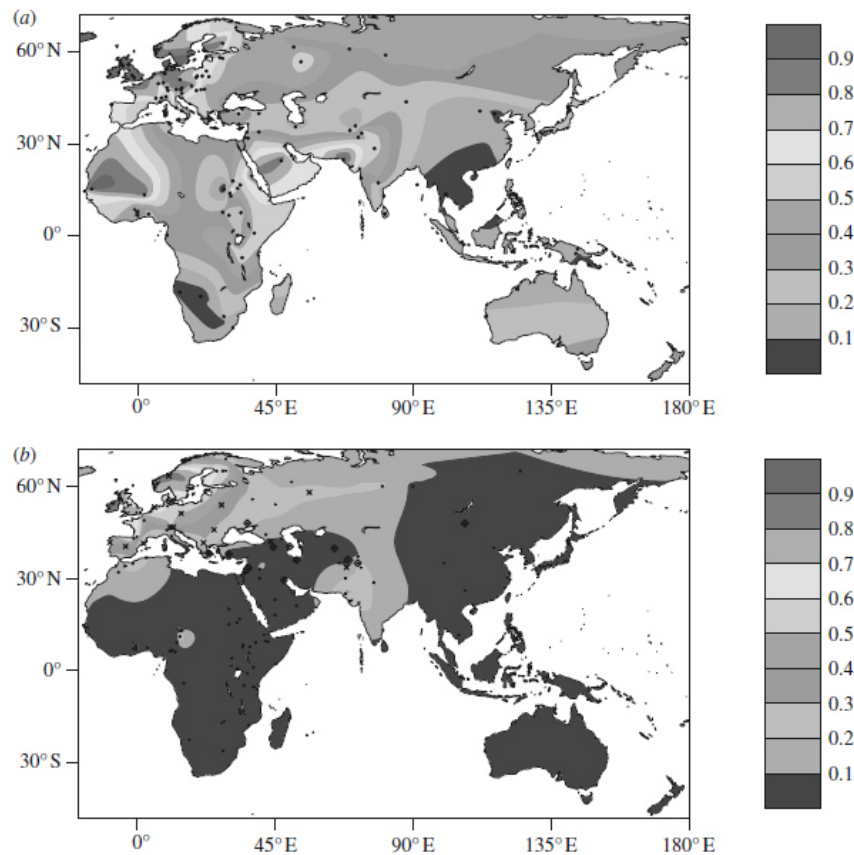


Figure 1. Interpolated maps of the distribution of LP and the -13910^*T allele in the 'old world'. (a) LP phenotype distribution. Data points (dots) were taken from the literature (see text and [14] for details). (b) Distribution of the allele -13910^*T , associated to LP. Dots represent sample data taken from a previous review [14,26–30]; crosses represent data for new locations not previously tested and diamonds correspond to locations where additional data have been added. Regularly updated frequency data are available at <http://www.ucl.ac.uk/mace-lab/GLAD/> website.

frequency of Somali people living in Ethiopia does not prevent them from drinking more than 500 ml of milk per day without any obvious discomfort [16]. This inter-individual variation of the amount of lactose tolerated by lactase non-persistent people may be a result of variation in the composition of the gut flora (particularly the presence of lactic acid bacteria) [16,43]. Also, fermented dairy products (i.e. yoghurt or cheese) contain less lactose, allowing consumption by non-persistent individuals without any of the expected symptoms [43].

Two contrasting theories have been proposed to explain the co-distribution of LP and dairying practices. The *culture-historical* hypothesis [11,12,44] argues LP developed, and was consequently selected, after milk production and dairy consumption spread. The opposing hypothesis (mentioned in McCracken [12] as the *reverse-cause* hypothesis) proposes that only populations whose frequency of LP was high enough adopted dairying. In other words, human groups were differentiated with regards to LP frequency by a process unrelated to milk consumption—through genetic drift [45]—before the invention of dairying. This view specifically assumes that drinking milk would not

necessarily have conferred any selective advantage. The culture-historical hypothesis is better supported by recent findings from archaeological research (see §1c for details). In particular, organic residues preserved in archaeological pottery provided evidence for the use of milk after 8500 BP in the western part of Turkey [40], a region where LP frequency is low today (figure 1a). It suggests that domestic animals were milked before LP arose or was present at appreciable frequencies.

(c) *The advantage of being lactase persistent*

Several explanations have been proposed for how and why LP may have been selected. For example, it may simply be that milk is a good source of calories, or specifically an important source of protein and fat. The milk production of a prehistoric cow has been estimated to range between 400 and 600 kg per weaning period. Even when the milk necessary for the raising of the calves is subtracted, some 150–250 kg remains [46]. This is almost equivalent to the calorie gain from the meat of a whole cow. Hence, over the years, milking may have resulted in a greater energy

yield than the use of cattle for meat. But it is likely that the benefits LP gave to early dairying populations extend beyond simply increasing the food supply or making more economic use of livestock. Furthermore, the specific benefits of dairying may have varied in space and time.

Strong selective pressures on LP may have been episodic and occurred only under certain extreme circumstances, such as drought, epidemic or famine. For example, milk would have represented an alternative food resource in between periods of crop cultivation. When no cereal food was available, for example between harvesting seasons or in periods of crop failure, LP individuals would have had an advantage. This is especially true for children after the period when lactase production is normally downregulated, a phase of life that shows an increased mortality according to osteological investigation of prehistoric skeletal collections [47,48]. In addition, Cook & Al-Torki [49] hypothesized that in regions where water was scarce, milk would have been used by pastoralist groups as relatively pathogen-free fluid. If by drinking fresh milk, lactase non-persistent individuals were at risk from the potentially dehydrating effects of diarrhoea under such conditions, selection may have been strong in lactase-persistent individuals.

However, this arid climate hypothesis is less likely to have been relevant in Europe, where LP frequencies are at their highest. The observed correlation between latitude and LP frequency in Europe led Flatz & Rotthauwe [50] to propose the *calcium assimilation* hypothesis. Calcium is essential for bone health and its absorption in the gut is dependent on the presence of vitamin D. While some food sources such as fish are rich in this vital nutrient, most people in the world produce the majority of their vitamin D photochemically in the skin through the action of UVB on 7-dehydrocholesterol. However, UVB exposure is insufficient to produce the required quantities of vitamin D in people living at high latitudes for much of the year. This is unlikely to have been a problem for pre-Neolithic European hunter-gatherers who would have had a vitamin D-rich diet through their consumption of marine foods [51], but may have been a problem for early agriculturalists. Additionally, it has been proposed that a fibre-rich diet, owing to high consumption of cereal grains, can lead to a reduction in the amount of plasma 25-hydroxyvitamin D₃ [52]. Thus, the consumption of milk has been suggested to have conferred an advantage to early LP farmers in regions such as the circum Baltic/North Sea area, where climatic conditions allowed high crop cultivation and consumption. Because milk contains small quantities of vitamin D and plenty of calcium, it can provide a valuable supplement at low-sunlight latitudes.

Bloom & Sherman [53] formulated the ecological dairying barrier hypothesis to complement the culture-historical model. They suggested that dairying, and therefore the evolution of LP, required environments that are favourable for raising milk-producing ungulates. Others proposed that the LP distribution is related to malaria [54]. They implied that LP is the ancestral phenotype and that lactase non-

persistence would have been selected in regions where the disease was frequent. However, this hypothesis was not supported by studies of lactase non-persistence prevalence in glucose-6-phosphate dehydrogenase deficient subjects from Sardinia [55,56]. In a contrasting hypothesis, it has been suggested that a milk diet would provide protection against malaria by impairing a part of plasmodia metabolism (folate synthesis) [52] and thus lead to selection for LP. Pointing to the tight relation between dairying and LP, it has also been hypothesized that milk drinking was a privilege restricted to some individuals in highly hierarchical societies and that it spread as prestige class behaviour [13,44,57]. (For further discussion on the spread of a prestige-associated culture, see [58].)

A common feature of most populations with high frequencies of LP is a history of dairying activity [11]. The availability of fresh milk to some human groups has challenged their niche, thereby creating a potential genetic feedback for a need of continuous lactase expression throughout adult life. As they appear to be interdependent feedback processes, analyses of LP genetic and cultural diversity can hardly be conducted separately [13]. Hence, it is likely that the study of the process of when, how and why some populations kept and exploited ungulates will shed new light on our understanding of the distribution of LP. Indeed, archaeological data can be used to provide evidence for the presence of this cultural behaviour in past populations. For example, the analysis of milk residues [59] and the determination of kill-off patterns of animals on archaeological sites [60] constitute two archaeological methods that can inform on the emergence of dairying. Consequently, to fully understand the origins and evolution of LP, it is necessary to consider archaeological and archaeozoological research on the origins of domestication and dairying. In Europe, the spread of domestic animals is tightly associated with the diffusion of the Neolithic from the Near East.

2. LACTASE PERSISTENCE, THE NEOLITHIC TRANSITION AND THE HISTORY OF DAIRYING

(a) *The spread of the Neolithic*

Before 8400 BP, hunter-gathering was the only subsistence strategy in Europe, but by 6000 BP, when farming had spread over most of the continent (figure 2), it had become rare. The spread of farming into Europe was dependent on earlier developments in the Neolithic core zone of the Near East and Anatolia. Archaeologically, this process defined a new period referred to as the 'Neolithic revolution'. It is characterized by the presence of polished stone tools and pottery, a more sedentary lifestyle and the management and subsequent domestication of certain animal and plant species. These features are often characterized as the 'Neolithic package', and this term has been widely used to define Neolithic sites, even though all these features are not always present together at these sites.

Chronologically and geographically, the Neolithic culture spread from its original core zone to other parts of western Eurasia and simultaneously to north

Africa. There are two opposing models for the spread of farming into Europe [62]. One, the *cultural diffusion*, or *acculturation* model, favours the idea that local hunter-gatherers adopted Neolithic practices once they gained access to them from farming neighbours. The alternative *demic diffusion* model holds that the Neolithic was carried throughout Europe by the movement of farming peoples (and consequently their genes) spreading into the territory of foragers. It is highly unlikely that the Neolithic transition could be wholly explained by either of these models alone. The reality is likely to have been more complex and involved local heterogeneity of farming adoption processes [63].

Analyses of European genetic diversity have attempted to address the relative role of acculturation and migrations on the European gene pool during the spread of farming and yielded contrasting results, depending on the methodology employed, the loci studied and the proxies for ancestral source populations used [64–70]. Recently, Bramanti *et al.* [71] presented ancient DNA evidence for a genetic discontinuity between late hunter-gatherers and early farming populations, at the beginning of the central European Neolithic 7500 BP, thus providing support for a migrating farmers model in this region. A full Neolithic lifestyle was established within a few generations in this area and cultural contact between farmers and local foragers persisted for some time. But the way in which Mesolithic hunter-gatherers and the Early Neolithic farmers interacted and how this led to an environment peopled exclusively by farmers is still debated [10,63,72].

(b) Domestic animals associated with the Neolithic transition

Archaeozoological data on domestic animals have been particularly valuable in establishing a more precise picture of the spread of the Neolithic and the new subsistence strategies associated with it. This involves bone analysis (animal species, sex, age at death, morphology, presence of cutting and cooking signs [14], C direct dating and ancient DNA analyses) and placing this within their archaeological context. Morphological changes [73] and culling strategies [74] can be used to identify where and when wild animals started to become herded livestock. During the pre-pottery Neolithic B phase (PPNB) in the Neolithic core zone, several human groups started to manage wild animals at approximately the same time, in different places. After a period of hundreds or even thousands of years, this process finally resulted in phenotypes characteristic of domesticates. These domestication-associated phenotypes illustrate one of the consequences of human cultural niche construction on the evolution of other species [3,8,10,75].

In the context of the Neolithic, the oldest evidence for domestication is for goat and sheep (11 000 BP) followed by pig and cattle (10 500 BP) [73,74,76,77]. These domesticates soon spread from the Neolithic core region to a large part of the Near East, including Cyprus [77,78] and central Anatolia. However, true animal husbandry as a major economic activity in the

Near East only began during the 10th millennium BP. Subsequently, but not earlier than 9000 BP, these domesticates spread into western and southern Anatolia [72,79]. From there, the spread of domesticates together with husbandry techniques followed two main routes (figure 2): (i) the Mediterranean route via the Aegean and Adriatic Seas, then moving further west to southern Italy, the Tyrrhenic Islands, southern France and the Iberian Peninsula; and (ii) a Danubian route via the Balkan Peninsula, further to the south-western part of central Europe and finally into central and northern Europe. The two routes might have met in Greece, in the Rhine valley and in the northwest of the continent before crossing to the British Isles [80].

Domestic goat (*Capra aegagrus*) and sheep (*Ovis aries*) must have been introduced to these areas by humans, as there is no evidence of indigenous wild progenitors in Europe. Ancient DNA studies indicate that cattle (*Bos taurus*) were also imported from Anatolia and, once in Europe, did not mix substantially with European wild cattle, the aurochs (*Bos primigenius*) [81–83]. This is probably because humans managed to keep these two forms of cattle reproductively separate, although substantial size differences and other consequences of domestication may also have limited interbreeding. In contrast, while domestic pigs seem to have been introduced into Europe from the Neolithic core zone, ancient DNA evidence indicates that they mixed substantially with local wild boar [84].

In terms of dairying, the further development of herd structure after the arrival of early domesticates in Europe is crucial. Indeed, neither the Early Neolithic in the Balkans (8200–7500 BP) nor the earliest Neolithic in central Europe (Linearbandkeramik, 7500–7000 BP) yield clear archaeozoological signs of highly specialized dairying. However, in the following periods, especially in central Europe, there is evidence of a progressive increase in herding associated with dairying, i.e. prevalence of female over male animals. This particularly holds true for cattle and goat [46]. Similarly, in southeast Europe, a transition to a more specialized dairying economy becomes increasingly apparent as early as the 7th millennium BP. It should be noted that it is very likely that dairying was practised long before this period [40,42] and may have represented a means of managing wild animals as early as the beginning of the domestication process. However, it did not become a substantial economic factor before the 7th millennium BP in most parts of Europe.

(c) Evidence of the consumption of dairy products

In addition to faunal remains, the detection of dairy fats associated with archaeological pottery is a powerful line of evidence for dairying activities in prehistory [59,85]. Investigations of organic residues in archaeological pottery have revealed a wide range of compound types, including dairy fats [85]. Using this method, the processing of milk has been identified in the western part of the present day Turkey as early as 8500 BP [40]. Similarly, evidence for the use of dairy products has been shown in Neolithic sites of

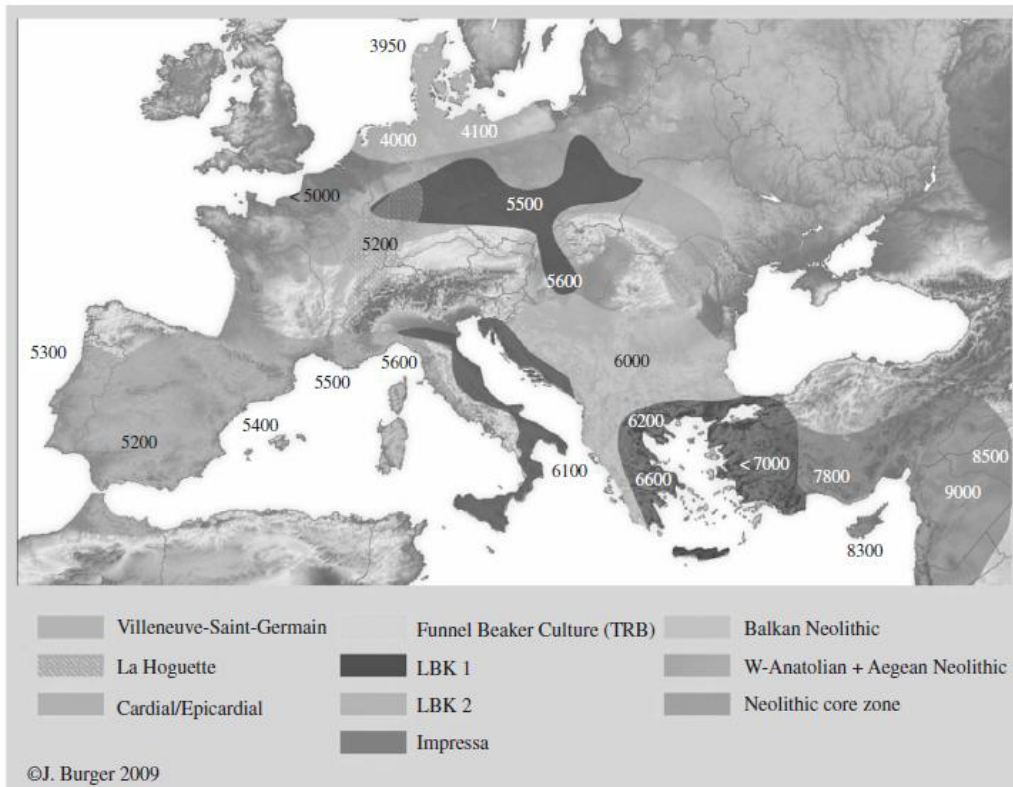


Figure 2. Arrival dates and approximate geographical expansions of defined Early Neolithic cultures from [61].

Romania and Hungary around 7900–7450 BP [39], Britain around 6100 BP [86] and Scotland around 3000 BP [87]. Thus, the archaeozoological and residual lipid data clearly indicate that dairying was practised early in Neolithic Europe. However, ancient DNA data from central and northern Europe suggest that LP frequency was low during this period [88]. Thus, we may hypothesize that Early Neolithic people, among whom LP was rare or absent, initially practised dairying in south-eastern Europe and later migrated towards central and northern Europe, an area inhabited by foragers that occupied a different niche [10].

Indeed, stable isotope studies of bone collagen show that while hunter-gatherers largely relied on marine food, the farmers' diet, even on coastal sites, was mainly terrestrial [51]. However, other authors found no evidence of this switch in diet [89–91]. This may be because the localization of hunter-gatherers compared with farmer sites is biased towards coastal regions [90,92], or there may be other methodological issues [91,92]. An additional possibility is that in regions with abundant aquatic resources, the process of subsistence change offered by Neolithic farmers would have been slower. For example, in Scandinavia, where marine resources are abundant, there is no direct evidence of a sudden dietary transition [90]. In fact, instead of a replacement of hunter-gatherers by farmers, hunters from the Pitted Ware Culture (PWC) [89] coexisted for nearly 1000 years with the Neolithic Funnel Beaker Culture (TRB). However, it

should be noted that recent ancient DNA evidence indicates little genetic continuity between PWC hunter-gatherers and modern Scandinavians [93].

In summary, LP and the main LP-associated allele in Europe, -13910^*T , are found at highest frequencies in northwest Europe where dairying arrived latest (figures 1a,b and 2). A simple—phylogeographic—interpretation of the latter would be that LP first evolved in northwest Europe. But computer simulation studies have shown that when a population expands, the centre of distribution of an allele can be far removed from its location of origin [94,95]. This process is called 'allele surfing' and is thought to have occurred with the spread of farmers in Europe [65]. Furthermore, selection has—to an extent—shaped the distribution of LP [14,36,37], although it is unclear whether this selection was continuous or episodic, and whether it varied by latitude [50] or ecological zones [53]. It is therefore clear that to obtain a more complete picture of the coevolution of LP and dairying in Europe it is necessary to integrate cultural, demographic and selective processes. Computer simulation represents one of the most promising approaches to understand these processes because it can combine multiple sources of information. However, as with all modelling approaches, it is necessary to identify the key evolutionary parameters that have shaped the observed data. This is a non-trivial task when one considers the range of proposed hypotheses to explain the current distribution of LP in Europe

today. In the following section, we review three of the simulation studies that have deepened our understanding of the coevolution of LP and dairying in Europe.

3. REVIEW OF SIMULATION STUDIES

(a) *Aoki's model of lactase persistence and niche construction*

The coevolution for LP and dairying simulated by Aoki [96] captures many of the features of niche construction, as later proposed by Laland *et al.* [5]. The evolution of populations, made up of four compound phenotypes (two genetic: LP versus non-LP individuals and two cultural: milk users versus non-users), was simulated. The compound phenotype of being an LP milk user was selectively advantageous (fitness of 1), whereas the other three phenotypes were assigned a selective value of $1 - s$ (where s is the coefficient of selection). The effective population size (N_e) assumed was 100 individuals. At each generation, the model considered: (i) random mating between individuals (the random combinations of alleles and cultural behaviour from a parental generation to the next), (ii) the transmission of the cultural trait occurred with distinct probabilities of becoming a milk user, i.e. $f(y)$ for an LP individual and $g(y)$ for a lactase non-persistent individual, and (iii) random sampling of the individuals after the action of selection. At the end of the process, a correlation between the fixation of both the LP trait and the cultural behaviour was measured, and an average time until fixation of LP was calculated. This study addressed three questions: (i) if the evolution of both LP and milk consumption is mutually dependent, what would the correlation between their frequencies be? (ii) Does the type of selection, either strictly genetic or culturally induced, produce differences in the rate at which an allele reaches fixation? (iii) Do the frequency estimates of European LP (falling in the interval (0.05–0.70)) fit the hypothesis of a gene–culture coevolution process that started 6000 years ago?

Although this model did not take into account cultural diffusion from neighbouring populations, an interesting feature is the use of distinct probabilities for transmission of the culture of milk consumption according to the LP phenotype of an individual. A key result was that an incomplete correlation between LP and milk consumption frequencies is expected. This actually represents inter-individual variability in the non-persistence phenotype, and corresponds either to LP individuals that do not drink milk or to lactase non-persistent individuals that do drink milk. It explicitly shows that the gene–culture coevolution hypothesis is still credible even if the correlation between the genetic and the cultural traits is incomplete. This is in accordance with previous analytical gene–culture coevolution studies [7,97,98] which have notably shown that genotype–phenotype correlation can be reduced by a factor depending on environmental and selection variances [97].

Another observation drawn from this model is that the change in LP frequency is slower when the selection is conditioned by culture. This means that in order to explain such high frequency of LP in

north-western Europe, we must consider that either every LP individual actually did drink milk, or that other processes have been involved to generate this distribution. The author consequently suggested that to be able to detect such a change in allele frequency since the start of dairying (then considered to have occurred 6000 years ago), either the effective population (N_e) was relatively small (100 individuals as simulated in the study), or the selection coefficient in favour of the phenotype was very high (more than 5% if $N_e = 500$). Such a high value of s per cent falls well within the confidence intervals ((1.4–19%) and (1–15%)) of recent studies ([20,36], respectively) inferred directly from genetic data. However, lower values of the selection coefficient may be expected to fit the observed LP pattern, as it is now known that the start of dairying was much earlier than 6000 years ago [40], leaving more time for the genotype to be selected. In addition, both genetic and archaeological studies point to the importance of the demographic process when studying the Neolithic diffusion in Europe [99,100], whereas Aoki's simulations were performed with a constant population size of 100 individuals. Moreover, demographic expansion has been shown to have a potentially dramatic effect on the diffusion of new mutations [94,95,101]. Hence, we may expect that the assumption of a constant population size could lead to an overestimation of the rate of frequency change and of the selection coefficients.

(b) *Spatial variation in selection intensity*

While the selection coefficients explored in Aoki's model were assumed to be constant, in a recent study, Gerbault *et al.* [18] modelled a geographical structuring of selection pressure by latitude, thereby testing explicitly the calcium assimilation hypothesis [50]. The evolution of a dominant allele associated with LP was simulated in four Near-Eastern and 22 European populations since the Neolithic transition in their respective regions, according to two demographic models: cultural diffusion and demic diffusion [63]. Each of these models was tested using either a constant selection coefficient or a selection coefficient varying with latitude. Thus, a total of four scenarios were tested combining the two selection models and the two demographic models. The program used (called SELECTOR) models four parametrized processes: (i) random genetic drift, (ii) logistically regulated population growth, (iii) positive selection varying between 0 and 3 per cent, and (iv) time elapsed since the onset of the Neolithic. To simulate the cultural diffusion model, the initial LP allele frequency was set at 1 per cent in all 26 populations and evolved according to the above processes. One important assumption is thus that the LP-associated allele was already present in Europe before the Neolithic but at a low frequency (1%). Under the demic diffusion model, three populations were used as sources in the Near East (Lebanon, Syria and Iran). The remaining populations, including the fourth Near Eastern population (Cyprus), were populated by sampling from neighbouring populations along the presumed route of the spread of farming, and at

the times indicated from archaeological data [41]. A maximum-likelihood test was performed to evaluate independently in each sample what selection coefficient best fitted the observed data (the LP frequency for each of the 26 populations). Moreover, the four simulated scenarios were formally compared using an approximate Bayesian computation (ABC) approach [102] based on LP frequencies within samples [103].

In this study [18], the scenario that gave the highest relative probability of obtaining the observed data was the demic diffusion model combined with a selection coefficient varying with latitude. Indeed, according to this model, genetic drift alone can explain frequencies as low as those observed in southern Europe, as previously suggested by Nei & Saitou [45]. However, in northern Europe, positive selection coefficients were required to drive LP frequencies to their observed values (figure 3). This result supports the calcium assimilation *hypothesis*. A complementary explanation for increased selection strength at higher latitudes is that the lower temperatures would allow milk to remain fresh for longer [44,57]. If this applies, LP would illustrate a specific case of niche construction where localized access to a resource is variable [5], i.e. fresh milk. Indeed, niche construction modelling [104] has shown that in such a situation a strong association between a cultural and a genetic trait is expected, as is observed for LP and dairying in northern Europe.

Even though the model of Gerbault *et al.* [18] does not simulate a wave of advance, it illustrates the importance of demography, since the demic diffusion model with variation in the selection coefficient was far more likely (99.1%) than the cultural diffusion model (0.9%). The selection coefficients estimated range between 0.8 and 1.8 per cent, and the authors suggested that if the simulations were closer to a wave of advance model, this range might have been lower. It should be noted that these estimates are not constant over all the populations considered, as under the best-fitted models the selection coefficient varied latitudinally and approached zero in southern Europe.

(c) *Demic and cultural diffusion of farming*

In a more complex spatially explicit model, Itan *et al.* [105] simulate the spread of farmers from the Near East to Europe over the last 9000 years, taking into account potential interactions with hunter-gatherers. The geographical unit of this simulation was a deme, and in total the simulated world was made of 2375 land demes, each containing three interacting populations: hunter-gatherers, dairying farmers and non-dairying farmers. At each generation, each population underwent a succession of seven processes: (i) logistically regulated population growth, in which each deme had a fixed carrying capacity determined by climatic and elevation factors. The carrying capacities of dairying and non-dairying farmers were equal, whereas those of hunter-gatherers were 50 times smaller [106,107]. (ii) Unidirectional migration process modelled as a stochastic Gaussian random walk from one deme to another (a process equivalent

to demic diffusion). (iii) Cultural diffusion, where a proportion of individuals from one culture 'converted' to one of the two other cultural groups, based on the relative 'dominance' of the other groups in the focal and eight surrounding demes. (iv) Intra-demic gene flow between different cultural groups within a deme. (v) Inter-demic gene flow between neighbouring demes belonging to the same cultural group. (vi) Selection acting on an LP allele only in the dairying farmers cultural group. (vii) Drift of the LP allele in all cultural groups in all demes, modelled as a binomial sampling process. The LP-associated allele was set to appear in a randomly chosen location when the population size of dairying farmers in this deme reached a critical value (20 individuals). This ensured that the LP-associated allele appeared on, or near, the wave-front of dairying farmer diffusion. Its frequency was updated according to the evolution of a dominant allele with parametrized selective advantage [108], which remained constant in each simulation. This selection in turn drove additional increases (over and above the logistically regulated population growth) in the population sizes of dairying farmers [108].

After the simulations, the authors estimated parameters of the model using ABC [102] with a particular focus on two related questions: (i) when and where in Europe did LP-dairying coevolution begin, and (ii) what factors drove the spread of LP to get the observed European pattern of LP frequencies? Simulations were fitted to both observed $-13910 \pm T$ frequencies at 12 European locations and the corresponding dates of arrival of farmers [41] at 11 of these locations. Both the dates estimated (between 6256 and 8683 years BP) and the geographical area identified (the region between central Europe and northern Balkans—figure 4) for the origin of LP-dairying coevolution correlate well with the time and location of the emergence of the Linearbandkeramik (LBK) culture (figure 2). The LBK is recognized to have been the direct forerunner of a Neolithic cattle-based economy [109], whereas on the Mediterranean Neolithic sites, faunal assemblages are more variable in composition and in some places dominated by sheep and goat [42,46,109]. Indeed, cows provide quantitatively more milk than sheep or goats, allowing cattle-based farmers to have a better supply, thereby supporting a stronger selection for LP. Even though these origin time and location estimates were not independently derived, as simulations were conditioned on known farming arrival dates [41], recent results from spatially explicit niche-construction modelling [9] corroborate this hypothesis. In this latter model [9], local mating of niche constructors (i.e. LBK-economy distribution and diffusion) has the effect of increasing the local concentration of the resource (i.e. fresh milk), thereby generating stronger selection in favour of the resource-dependent trait (i.e. LP).

Indeed, from the study of Itan *et al.* [105], the selection coefficients necessary to explain the $-13910 \pm T$ allele frequency distribution in Europe were estimated to range from 5.2 to 15.9 per cent. Although very high, this range falls within the range estimated (1.4–19%) from molecular data [36], but shows less overlap with the range estimated by Gerbault *et al.* [18].

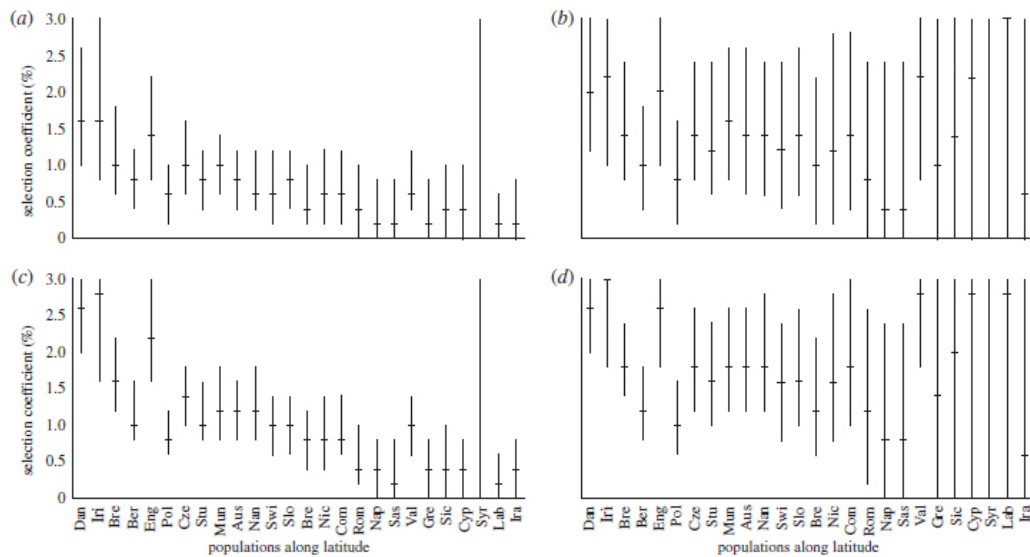


Figure 3. Scenarios simulated in [18] and selection coefficients required to fit the observed estimates of LP frequencies (taken from [18]). Bars represent the 95% confidence interval of the selection coefficient estimated for the population and the central point is the MLE (maximum-likelihood estimate). Populations are ordered from the highest to the lowest latitude: Danish (Dan), Irish (Iri), German from Bremen (Bre), German from Berlin (Ber), English (Eng), Polish (Pol), Czech (Cze), German from Stuttgart (Stu), German from Munchen (Mun), Austrian (Aus), French from Nantes (Nan), Swiss (Swi), Slovenian (Slo), Italian from Brescia (Bre), French from Nice (Nic), Spanish from Santiago de Compostela (Com), Italian from Roma (Rom), Italian from Napoli (Nap), Sardinian (Sas), Spanish from Valencia (Val), Greek (Gre), Sicilian (Sic), Cypriot (Cyp), Lebanese (Leb) and Iranian (Ira) (doi:10.1371/journal.pone.0006369.g002). (a) Demic diffusion, gene-culture coevolution; (b) demic diffusion, calcium assimilation; (c) cultural diffusion, gene-culture coevolution; and (d) cultural diffusion, calcium assimilation.

However, as the selection strength inferred by Itan *et al.* [105] applies to less than half of the overall population (dairying farmers), the population-wide selection coefficient is likely to be around half of the value reported. A key difference between the two studies is that Gerbault *et al.* [18] explicitly modelled, and found support for, a latitudinal effect on LP selection coefficients [50] while Itan *et al.* [105] did not. Even though it is difficult to separate the effects of selection and demography in their model (as demographic parameters and the selection coefficient were estimated simultaneously), Itan *et al.* [105] argued that such a latitudinal effect is not *necessary* to explain the higher observed frequencies of LP in northern Europe. Indeed, they found that without any assumption of a latitudinal effect on selection, their model under the estimated parameters predicted higher LP frequencies in northern Europe than observed, and typically lower frequencies in southern Europe than observed. Thus, the addition of latitudinal effect on selection should drive an even poorer fit to the LP data and so could be thought of as having 'negative explanatory power'. However, it should be noted that Itan *et al.* [105] did not explicitly model a latitudinal effect on selection, and so have not formally rejected it.

Another key difference between both studies is the values estimated for the selection coefficient, which are larger in Itan *et al.* [105] than in Gerbault *et al.*

[18]. In the study of Itan *et al.* [105], the best fit was obtained when the allele was first selected in dairy farmers, in central Europe/the northern Balkans, and subsequently spread into neighbouring regions. High selection coefficients were thus required to obtain the high frequencies currently observed in north and central Europe in a relatively short period of time. In the model of Gerbault *et al.* [18], the allele was assumed to have reached non-negligible frequencies before it had spread into central Europe as it was already present in the Near East at the onset of the Neolithic transition. Hence, while selection was required, this was to a lesser extent than if it had started in central Europe. There is a potential 'trade-off' between selection intensity and the timing and location of origin of the gene-culture coevolutionary process, and in future work, combinations of values for these parameters will bring insights into the means of diffusion not only of LP but of other genetic traits as well.

4. DISCUSSION AND PERSPECTIVES

(a) Simulation overview

Although simulation studies are unlikely to recover the precise evolutionary history of LP and dairying, they have two main advantages over purely genetic or purely archaeological studies. First, they allow the integration of information from multiple sources

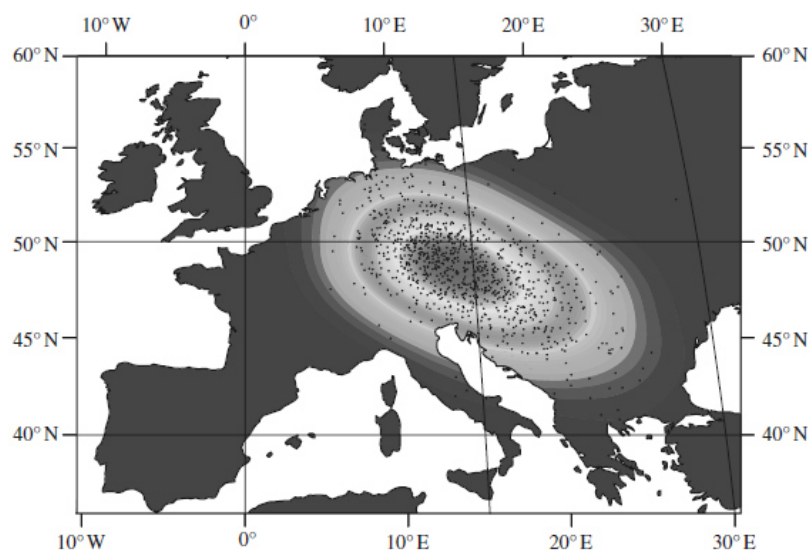


Figure 4. Approximate posterior density of region of origin for LP—dairying coevolution (taken from [105]). Points represent regression-adjusted latitude and longitude coordinates from simulations accepted at the 0.5% tolerance level. Shading was added using two-dimensional kernel density estimation (doi:10.1371/journal.pcbi.1000491.g003).

(genetics, archaeology, ecology). Second, they provide a formal comparison of alternative scenarios, according to the parameters taken into account. Many combinations of parameters can be tested, and extreme scenarios (such as cultural or demic diffusion) can be evaluated statistically, and potentially excluded. Furthermore, ABC methods [102,103] have proved to be very useful when evaluating the fit of observed data on complex scenarios tested, adding power to simulation studies [18,105]. Clearly, simulations performed at the continental scale help to understand general features and processes of the European Neolithic transition, and to explore alternative possibilities that may have led to observed patterns of genetic diversity.

(b) *Demography and niche construction*

The above simulation studies of LP/dairying coevolution, as well as ecological and archaeological information, suggest that selection on LP is unlikely to have been constant over time and space during its spread throughout Europe, and show that the role of demography in its diffusion cannot be ignored. The extent to which combinations of these two phenomena have shaped the LP distribution as it is observed in Europe remains unanswered. While Itan and colleagues [105] used a complex demographic model, the population growth was logistically regulated for each group, with no direct consequence of the growth of one group on the growth of the others (i.e. competition between cultural groups for land resources). But as almost no hunter–gatherers survive in Europe today, it is logical to assume that niche modification by farmers may have had an effect on the distribution of hunter–gatherers that led ultimately to their disappearance (because of the distinct abilities

of both populations to niche construct [10]). As an adaptation of the model of Itan *et al.* [105], an alternative dynamic could be proposed to investigate how demographic constraints may have affected the diffusion of the trait. Inclusion of density-dependent competition, as implemented by Currat & Excoffier [110], should bring new insights into the effect of these demographic processes. This improvement would allow regional variation in the simulation of the Neolithic spread, with a higher carrying capacity of hunter–gatherer populations in areas where marine resources are abundant, thereby potentially changing the competition dynamics between hunter–gatherers and farmers. This would also enable account to be taken both of localized resource availability and spatially structured populations for investigating the evolution of the association of LP (i.e. the resource-dependent trait) and dairying (i.e. the niche-constructing practice) [9,104]. This may be relevant for the co-habitation of hunters from the PWC [89] with the TRB farmers, who coexisted for nearly 1000 years in Scandinavia. Further analyses assessing this co-habitation would be appropriate to explicitly evaluate how the co-influences of genes, environment and pre-existing culture affect differential transmission of Neolithic cultural forms [111].

The spread of LP is a good example of niche construction for two reasons. First, because human groups who started to drink milk modified their pre-existing selection pressure, thereby generating an evolutionary feedback that may have advantaged some individuals against others in certain environmental conditions. Second, by perpetuating this behaviour from the Neolithic to modern days, the coevolution of LP and dairying illustrates how culture can affect the genetic diversity of human populations. That is why niche construction factors should be considered

when studying human adaptation. Gene–culture co-evolution studies have shown that both genes and culture exert distinct, but interacting, influences in the evolution of human phenotypes [98,111]. More precisely, cultural processes have been shown to alter the outcome expected under purely genetic transmission [7,97,98]. Even though dairying culture has been taken into account, it should be noted that the effect of differential cultural transmission has not been explicitly modelled in the three simulation studies described above. It is thus expected that the combination of demographic information and differential cultural and genetic transmission influenced by selection would enlarge the possible evolutionary conditions that may have shaped the worldwide LP distribution observed today.

(c) *Migration and environment*

Other environmental parameters are likely to have affected the distribution of the culturally distinct populations, and the speed of the Neolithic transition. Indeed, niche-construction studies have shown that environmental variables should not be seen as static but as dynamic features that change according to climate and human activities [5], thereby modifying the selection pressure to which humans are subjected [35]. For example, Itan *et al.* [105] took into account environmental factors to determine different carrying capacities according to the economy type of the populations. In addition to this, other environmental pressures, such as vegetation cover and natural water access, may have conditioned the movement of farming populations with their domesticated animals [57]. These environmental variables can allow the modelling of potential regional variation in the spread of the Neolithic, as suggested by archaeological studies [112]. This will have an initial advantage of bringing a better understanding of the effect of the environment on the spread of agriculture and farming. Then, the relative importance of environmental variables that influence migrations can be evaluated. However, it should be noted that increasing the number of parameters in a model is generally to be avoided unless necessary. In particular, because the more complex a model is (the closer to the reality it is), the more difficult it becomes to interpret, as isolating the effects of different processes, and parameters, can be extremely complicated. Furthermore, the parameter space of a complex model can be very difficult to explore by simulation.

(d) *The importance of an interdisciplinary approach*

While selection on LP has been inferred, it has not been shown directly [113]. Other pleiotropic factors may be involved that affect the ability to digest milk on the one hand or that affect selection on *LCT* on the other. What is clear from the study of LP-associated genetic variants is that it has emerged independently in different regions of the world [14,20,23,31,32,114]. While some alleles show evidence of strong directional selection, soft selective sweeps [115]—whereby ancestral genetic variation is associated with different adaptive substitutions

[116]—have been invoked [16] to explain how several alleles can be maintained in a population through mutation and migration processes. It would be of interest to assess whether such a process applies more widely than in Africa and the Middle East. Ongoing research on the *LCT* gene and the regulation of its expression, and further sampling, with associated sequencing and phenotyping will be necessary to give a more complete picture of the structure of LP-associated genetic diversity [15]. Additionally, archaeological assemblage studies will provide useful information on the time and location of the development of milk-drinking behaviour. Finally, simulation studies can assess different possible scenarios by taking into account information obtained from different fields of study, particularly by employing ABC methods [102,103]. LP evolution will be better understood by combining data from these different fields, and by generating expectations of different data types in simulations. More broadly, the study of LP evolution is likely to shed light on the interactions and relative contributions of distinct processes involved in shaping human variation.

5. CONCLUSION

The coevolution of LP and dairying appears to be not as straightforward a process as it may have first seemed. While several alleles seem to be associated with LP, the pattern of LP in Europe appears peculiar, as a single variant (-13910^*T) has been identified as explaining most of the modern distribution of LP [15]. In this continent, the spread of dairying-associated practices goes back to Early Neolithic periods, and it is likely that archaeological studies will soon give a more precise picture of the early development of dairying in Europe. Archaeology notably underlines how complex the migration processes may have been. It may not be surprising then, even though the models were different, that simulation studies on the spread of LP in Europe highlight the importance of demography on the distribution of the -13910^*T allele. Some of these simulation studies also hint that selection may not have been constant over time and space. It thus appears that, in order to disentangle the effects of selection from those of demography on the distribution of both LP phenotype and genotypes, there is a need to better understand spatial patterns of genetic variation under neutrality [66,101,117,118]. In turn, the extent to which LP-associated genetic variation can be explained by these patterns will determine what can be said about the adaptation process [119]. Inference on the coevolution of LP (and its associated alleles) and dairying is likely to provide a useful framework for future studies on the evolution of other adaptive traits in which niche construction is invoked.

We thank two anonymous referees for their comments. P.G. and A.L. are funded by an EU Marie Curie FP7 Framework Programme grant (LeCHE, grant ref: 215362-2). Y.I. was funded by the B'nai B'rith/Leo Baeck London Lodge and Annals of Human Genetics scholarships. We also thank the AHRC Center for the Evolution of Cultural Diversity (CECD) and the Center for Mathematics and Physics in

the Life Sciences and Experimental Biology (CoMPLEX), UCL, for supporting this research.

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